University of Alberta

Genetic Manipulation of Aspartate Aminotransferase Levels in *Brassica napus*: Effects on Nitrogen use Efficiency

by

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Abstract

Current farming practices include applications of nitrogenous fertilizers to increase plant growth. These fertilizers are applied in excess to compensate for losses due to volatilization, leaching, and denitrification which, contribute to numerous deleterious health and environmental effects. One solution to curb the use of excess fertilizer is to develop nitrogen efficient plants that maintain a high yield when grown at reduced nitrogenous fertilizer rates. I have focused on developing these nitrogen efficient plants by over-expressing an aspartate aminotransferase (AAT) gene in a *Brassica napus* background. Thirteen independent transgenic lines containing a single insertion event were identified and advanced to the T_3 generation for characterization. One of these lines represents a putative nitrogen efficient genotype, accumulating higher shoot and root biomasses than control plants. The increases however, could not be replicated in a second experiment. Surprisingly, none of the lines showed a detectable increase in AAT activity despite evidence of transgene expression.

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List of Abbreviations

μmol photon/m^2·s	micromoles of photons per metre squared per second
μg	microgram
μg/ml	micrograms/millitre
μl	microlitre
AAT	aspartate aminotransferase
AS	asparagine synthetase
Asn	asparagine
Asp	aspartate
bp	base pairs
C	carbon
CaMV 35S	cauliflower mosaic virus 35S promoter
CAN	Canadian
cents/litre	cents per litre
Fd-GOGAT	ferredoxin dependent glutamate synthase
g	gram
Gln	glutamine
Glu	glutamate
GOGAT	glutamate synthase
GS	glutamine synthetase
ID#	identification number
Kan^R	kanamycin resistant
Kan^S	kanamycin sensitive
Kg/ha	kilograms per hectare
l	litre
Mg	megagrams
mg	milligram
mg/l	milligrams per litre
mg/ml	milligrams per milliliter
ml	milliliter
mM	millimolar
mmol	millimole
mV	millivolt

N	nitrogen
NADH-GOGAT	nicotinamide adenine dinucleotide dependent glutamate synthase
NiR	nitrite reductase
nM	nanomolar
N-o-1	double haploid line derived from *Brassica napus* cv. Westar
NR	nitrate reductase
NtE	nitrogen utilization efficiency
NUE	nitrogen use efficiency
NUEb	biomass nitrogen use efficiency
NUEg	grain nitrogen use efficiency
N/yr	nitrogen per year
QTL	quantitative trait loci
Tg	teragram
U	unit
UI	utilization index
UpE	nitrogen uptake efficiency

1. Introduction

1.1. The Green Revolution

An agricultural revolution began in the 1960's that has since become known as the green revolution. This revolution was initially characterized by the introduction of high-yielding, semi-dwarf varieties of wheat and rice (Lawrence, 2000; Khush, 2001; Evenson and Gollin, 2003; Hedden, 2003; Sakamoto and Matsuoka, 2004). These semi-dwarf plants had two major advantages over earlier varieties; they had a higher harvest index (Khush, 1999; Khush, 2001) and improved nitrogen (N) responsiveness (Coffman and Bates, 1993; Evenson and Gollin, 2003). The gains made in harvest index (grain weight : straw weight) resulted directly from the reduction in height of the semi-dwarf plants. This reduced the amount of straw produced by the plant and allowed the plant to allocate more energy and assimilate to grain production (Hedden, 2003).

Improved N responsiveness of semi-dwarf plants was an indirect cause of their short stature. Earlier varieties responded to high exogenous N concentrations by growing excessively tall (Sakamoto and Matsuoka, 2004). Consequently, the plants frequently lodged resulting in yield losses (Hedden, 2003). The short, stiff stalks of the semi-dwarf plants resisted lodging allowing farmers to take full advantage of nitrogenous fertilizers.

Cultivation of semi-dwarf varieties of wheat and rice in Latin America and Asia increased during the 1970's following their adaptation to local sub-tropical and tropical environments (Coffman and Bates, 1993; Khush, 1999; Evenson and Gollin, 2003). Year round cropping systems were later made possible in these environments with the introduction of early maturing, photoperiod insensitive plants that did not require

vernalization. Increased consumption of chemical inputs such as fertilizers (FAO data base, 2004) (Figure 1.1) accompanied the introduction of year round cropping of semi-dwarf cereal varieties. This resulted in an overly simplified agricultural system maintained in a nutrient rich state. The need to reduce inputs while maintaining yield however, is now recognized (Smil, 1999; Socolow, 1999; Tilman et al., 2002).

1.2.1. Anthropogenic Alteration of the Nitrogen Cycle

Many researchers believe that humans have increased the total amount of N being fixed on an annual basis (Vitousek, 1994; Galloway et al., 1995; Vitousek et al., 1997 Socolow, 1999; Vitousek et al., 1997; Smil, 1999). Current estimates for total anthropogenic N fixation are now approximately equal to the 140 Tg of N fixed per year by natural processes that include lightning (<10 Tg N/yr) and biological fixation by microorganisms in symbiotic relationships with higher land plants (between 90-130 Tg N/yr) (Table 1.1) (Galloway et al., 1995; Vitousek et al., 1997). The only unknown is the amount of N fixation that occurs in the sea; estimates for this value range from 40 to 200 Tg N/yr. Current estimates for marine N fixation are somewhat more constrained at 125 ± 50 Tg N/yr (Gruber, 1997). However, many authors neglect to include this in their models presumably because of a lack of understanding and the degree of uncertainty of marine N fixation. What is clear, though, is that humans have considerably increased the total amount of N fixed per year, particularly in terrestrial ecosystems. This increase is also highly localized but globally distributed such that it has significant impact on numerous ecosystems world wide (Matson et al., 2004).

1.2.2. Fertilizers and the Haber-Bosch Process

Until the advent of the Haber-Bosch process in 1908 by Fritz Haber and its industrialization in 1914 by Karl Bosch, there was no economically feasible method of fixing N (Frink et al., 1999). Nitrogen for agricultural use was derived by crop management techniques, such as including legumes in crop rotation, spreading of manures, or letting a field lay fallow (Smil, 2001; Galloway and Cowling, 2002). Deposits of guano and Chilean Saltpeter ($NaNO_3$) were also of importance before 1914, but sources of these "natural fertilizers" did not provide enough N to satisfy the demand and became increasingly expensive. The Haber-Bosch process provided a source of low cost N for agricultural use. Without this process the green revolution would not have had the impact of more than doubling global cereal production over the past 40 years (FAO data base, 2004). As a result, nitrogenous fertilizer use has increase 6.87 fold over a similar period of time (1961-1996) (Tilman, 1999). Although this increase accounts for a substantial proportion of the total anthropogenic N fixed today (95 Tg N/yr) (Socolow, 1999), it is not the only source.

1.2.3. Anthropogenic Nitrogen Fixation

Both combustion and legumes contribute to global anthropogenic N fixation. Combustion of fossil fuels however, not only fixes molecular N (N_2) in the atmosphere but also liberates previously fixed N in the fuel itself (Vitousek et al., 1997). Together, these processes account for ~ 20 Tg of biologically active N per year (Galloway et al., 1995; Vitousek et al., 1997). The remaining anthropogenic N fixed per year results from the increased acreage of leguminous crops planted globally (FAO data base, 2004).

Fixation occurs via a symbiotic relationship with nitrogen fixing bacterial genera such as *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* (Hopkins, 1999) and accounts for ~ 40 Tg N fixed per year (Galloway et al., 1995).

1.2.4. Remobilization of Fixed Nitrogen

The doubling of global cereal production during the Green Revolution was not solely due to the developments of modern varieties of wheat and rice, increased chemical inputs or their interaction. A small but important increase in total cultivated lands has occurred (FAO data base, 2004). Prior to cultivation the land is cleared of native flora, excess biomass is burnt and existing wetlands are drained (Vitousek and Matson, 1993). Together these activities volatilize and remobilize an estimated 70 Tg of previously fixed and stored N per year (Vitousek et al., 1997). Furthermore, wetlands are prime habitat for denitrifying bacteria that convert biologically active N such as nitrate (NO_3^-) to an inert form of N (N_2) under anaerobic conditions (DelGrosso et al., 2000; Keddy, 2000). The draining of wetlands removes this important N sink.

1.3. The Nitrogen Biogeochemical Cycle

Anthropogenic N fixation and re-mobilization have altered the global N cycle through the addition of biologically active N into the environment. To understand the effects caused by this alteration it is necessary to first understand the global N cycle. N_2 is converted into ammonia (NH_3) through a process termed fixation (Follett, 2001; Chapin et al., 2002). Lightning and bacteria, either free living or in symbiotic relationships with plants, are the only natural means of carrying out this process. The

majority of the NH_3 is then taken up by plants and microorganisms as ammonia (NH_4^+) and then assimilated into organic compounds. Consumption of plant or animal matter by animals converts previously assimilated N into other forms. Degradation, decay and mineralization of organic matter from plants, animals, and microorganism ultimately return NH_3 to the soil to begin the cycle anew.

NH_3 can also affix to negatively charged soil particles in its ionized form (ammonium; NH_4^+) or it can be oxidized into nitrite (NO_2^-) and NO_3^- progressively, processes known as micelle fixation and nitrification respectively (Follett, 2001; Chapin et al., 2002). The resulting NO_3^- can be used as a source of N for plants, similar to NH_4^+, or it can be denitrified. Denitrification reduces NO_3^- into nitric oxide (NO), nitrous oxide (N_2O), or N_2. The degree of reduction is dependent on the species of N and the environment in which the denitrification is occurring, but eventually N_2 is returned to the atmosphere, completing the cycle.

1.4.1. Environmental Impacts of Excess Nitrogenous Fertilizers: Eutrophication

Standard farm practices have always included the application of nitrogenous fertilizers to crop land (Smil, 2001). The introduction of cheap, industrially fixed nitrogenous fertilizers and the green revolution's N responsive cultivars however, modified the practice. A one time excess surface application, often early in the season, prior to seeding, is cheaper and less time consuming than alternative practices. Unfortunately, this method has significant environmental consequences.

Surface broadcast NH_3 based fertilizers, such as urea, can result in significant N losses (Smil, 1999; Mosier, 2002). These losses are due to volatilization of NH_3 and have

been reported to reach 60-70% of the N applied. Since the beginning of the green revolution, volatilization of NH_3 has increased 540% to 9 Tg N/ yr in 1990 (Mosier, 2001). Once in the atmosphere, NH_3 can travel hundreds of kilometers away, with the bulk of NH_3 deposited near the source (Ferm, 1998; Mosier, 2001). As a result, shifts in species diversity and abundance have occurred on lands that were previously N limited (Bobbink et al., 1998; Ferm, 1998; Socolow, 1999). These ecosystem changes in species composition or abundance result in the loss of ecosystem services that have been described as essential to human existence (see Tilman et al., 2002 for examples of ecosystem services).

Similar to the eutrophication of terrestrial environments by the application of excess NH_3 based fertilizers, the application of excess NO_3^- based fertilizers can eutrophy aquatic environments. However, unlike protonated forms of NH_3 that are usually bound to soil particles, NO_3^- is highly mobile in the soil substrate (Smil, 1999). If not immediately taken up by plants or microorganisms, 10% to 20% of the N applied can leach into ground water, depending on the source of N and the soil type. The contaminated ground water ultimately ends up in N limited coastal marine environments. Increased productivity and biomass of algae species in particular, accompany the NO_3^- influx causing a cascade of effects (Smith, 2003). The end result is an area of hypoxia or anoxia commonly referred to as a "dead zone". The most notorious "dead zone" occurs in the Gulf of Mexico. This area exhibits the loss of certain ecosystem services due to changes in species composition and abundance, analogous to eutrophied terrestrial environments (Rabalais et al., 2002; Donner, 2004).

Alternatively, both NO_3^- and NH_3 based fertilizers can enter the watershed if heavy rains and/or flooding occur at the time of application (Follett, 2001). Losses due to surface run-off have been estimated between 1% and 13% of the total applied N (Raun and Johnson, 1999). This N ultimately ends up in coastal, marine environments with similar effects to leached nitrogenous fertilizers.

1.4.2. Environmental Impacts of Excess Nitrogenous Fertilizers: Methemoglobinemia

Nitrate contamination of drinking water, from leaching or run off also poses a human health concern. Current acceptable limits for NO_3^- and NO_2^- contamination of drinking water are 50 mg/l and 3 mg/l respectively (WHO, 1998). Consumption of water with concentrations above these limits has been linked to methemoglobinemia; an anemic condition that infants under three months are particularly vulnerable to (WHO, 1998; Socolow, 1999).

1.4.3. Environmental Impacts of Excess Nitrogenous Fertilizers: Greenhouse Effect

Application of NO_3^- based fertilizers in wet conditions produce water logged, anaerobic soils rich in NO_3^-; conditions prime for denitrification to occur (Chapin et al., 2002; Mosier, 2002). As mentioned in section 1.2.4., species such as N_2, N_2O and NO result from this denitrification. Global agricultural emissions of N_2O alone have doubled between 1950-1996 to over 6 Tg N/yr (Mosier, 2001). This increase has contributed significantly to the increase in the greenhouse effect due to the stability of N_2O in the atmosphere and the effectiveness with which N_2O absorbs infrared radiation. One

molecule of N_2O absorbs 200 times more infrared radiation than a molecule of CO_2 (Socolow, 1999).

1.4.4. Environmental Impacts of Excess Nitrogenous Fertilizers: Ozone Concentrations

Unlike N_2O which is relatively inert, NO is highly reactive (Cobb and Braman, 1995). In the troposphere it reacts to produce tropospheric ozone, a major component of air pollution (Socolow, 1999). In the stratosphere NO acts as a catalyst to destroy stratospheric ozone. The source of troposphere and stratosphere NO however, is different. Tropospheric NO is produced by a number of processes including fossil fuel combustion, biomass burning, and agriculture (Mosier, 2001). Agriculturally, NO is produced directly through denitrification and/or nitrification, depending on environmental conditions (Vitousek and Matson, 1993). This NO is volatilized above agricultural lands and contributes significantly to tropospheric ozone concentrations in rural areas where the landscaped is dominated by agriculture (Davidson et al., 1998). In contrast, stratospheric NO is produced indirectly via the break down of stratospheric N_2O by high energy ultraviolet light (Socolow, 1999).

1.5. Economic Cost of Excess Nitrogenous Fertilizers

Industrial production of nitrogenous fertilizers requires high temperatures and a source of hydrogen, both achieved through the use of natural gas (Galloway et al., 1995; Smil, 2001). Between 1989 and 2003 the price paid by Canadian producers for natural gas has increased from 9.02 cents/litre to 26.68 cents/litre (Statistics Canada, 2004). This increase in natural gas cost has had a direct impact on the cost of nitrogenous fertilizers.

In Canada, the price of urea has increased from a low of $(CAN) 206/tonne in 1971 to a high of $(CAN) 834/tonne in 1995 (FAO data base, 2004). Similar trends exist for ammonia in Canada and for urea and ammonia in the United States (Figure 1.3).

Despite this increase, nitrogenous fertilizers are still an affordable commodity (Raun and Johnson, 1999). However, Raun and Johnson (1999) point out that a 1% increase in cereal crop-plant nitrogen use-efficiency could reduce the amount of N fertilizers applied globally by 4.9×10^5 Mg per year resulting in an annual global saving of $2.3 billion. Considering that N fertilizers are currently applied in excess, it is rational to assume that this savings is immediately available through a 1% reduction in applied N without an accompanied reduction in yield. This reduction has not happened however, presumably because a one time application of excess nitrogenous fertilizer early in the season is less time consuming and costly than matching nitrogenous fertilizer application to the N requirements of a crop.

1.6. Nitrogen Efficient Cultivars

How efficiently a plant uses a particular nutrient was originally defined as a plant's biomass per unit of nutrient contained within that biomass (Steenbjerg and Jakobson, 1963). For N, this would be referred to as nitrogen use efficiency (NUE). It was later demonstrated that this value did not adequately differentiate efficient plants from non-efficient ones, particularly in complex systems, because it failed to give adequate consideration of the biomass component and did not take tissue nutrient concentration into consideration (Siddiqi and Glass, 1981). Siddiqi and Glass proposed

to redefine nutrient use efficiency as a plant's biomass per nutrient concentration within that biomass and renamed the term the utilization index (UI).

Unfortunately, this definition is of little agronomic value for two reasons. First, UI does not take into account the amount of nutrient available in the soil. As such, UI does not allow for a cost benefit analysis of increasing yield through fertilization. Second, UI does not account for crop yield. Generally, yield is a higher priority than biomass to producers because it represents the marketable product.

Grain nitrogen use efficiency (NUEg), as defined by Moll et al. (1982), addresses both of these concerns. NUEg is defined as the yield per unit N applied and is the product of N utilization efficiency (NtE), yield per unit of total N in the plant, and N uptake efficiency (UpE), total N in the plant per unit applied N. It follows then, that an increase in NUEg can result from an increase in yield without a proportional increase in N fertilizer rate (scenario 1). An alternative scenario (scenario 2), results in a similar increase in NUEg but through a decrease in applied N without a proportional decrease in yield.

By plotting the relationship between yield and applied N at a number of nitrogenous fertilizer rates, a nutrient growth response curve can be generated (Figure 1.4-6). Interestingly, the slope of the resulting function describes NUEg and any increase in the slope indicates an increase in NUEg. As such, a wide array of growth curves could represent plants that are N efficient. However, only a subset of these growth curves, described by scenario 2 above, actually represent N efficient varieties that could potentially reduce the amount of nitrogenous fertilizers applied to arable land.

1.7.1. Strategies for Developing Nitrogen Efficient Cultivars: Plant Breeding

One strategy for developing N efficient cultivars is traditional breeding. Studies with several crop species indicate that genetic variation in NUE, NUEg, and the components of NUEg exist in current germplasm. Wu and Tao (1995) showed that NtE and UpE significantly varied between rice genotypes and N fertilizer rates. Data obtained from tobacco cultivars have shown similar results and included NUEg variability (Sisson et al., 1991). In current European maize breeding lines, Presterl et al. (2003) found significant genotypic variance for grain yield and dry matter content at both low and high nitrogenous fertilizer applications. *Brassica napus* also showed variability in NtE and UpE (Yau and Thurling, 1987) and diverse Sorghum varieties exhibited significantly different NUE (Gardner et al., 1994) and NUEg (Buah et al., 1998) both between varieties and nitrogenous fertilizer rate. Data from wheat (Orti-Monasterio et al., 1997) and barley (Gorny and Sodkiewicz, 2001; Sinebo et al., 2004) have shown similar results for NUEg and NUE respectively. This variability suggests that breeding programs with the goal of increasing NUEg in agronomically important crops may be feasible with the current germplasm.

The recent identification of quantitative trait loci (QTL) involved in various aspects of N metabolism and components of NUE and/or NUEg in *Arabidopsis* (Rauh et al., 2002; Loudet et al., 2003), maize (Agrama et al., 1999), barley (Mickelson et al., 2003) and rice (Yamaya et al., 2002) should also assist breeding programs. Plants containing QTLs that contribute to NUEg, can be selected at an early stage by identifying those plants containing molecular markers linked to the respective QTLs. This type of

marker assisted selection, in combination with traditional breeding, could facilitate the creation of NUEg crop species.

1.7.2. Strategies for Developing Nitrogen Efficient Cultivars: Genetic Engineering

The other approach available for developing N efficient cultivars is through genetic engineering. Many groups have attempted to increase NUE and/or NUEg in a variety of species through the introduction of specific transgenes (see Good et al., 2004). The choice of specific promoter/transgene combination is often related to previous knowledge of plant nitrogen metabolism and/or choice of available cloned promoters and genes. Alternatively, candidate genes could be chosen through the fine mapping of a specific QTL in order to identify the gene responsible for the effect of the corresponding QTL. Cytosolic glutamine synthetase (GS) has already been observed to co-localize with QTLs implicated in N metabolism in *Arabidopsis* (Loudet et al., 2003) and rice (Yamaya et al., 2002). Cloning of the gene encoding GS allows GS activity to be modified *in planta* in an attempt to increase plant NUEg; work that has already been attempted (section 1.8.). The identification and subsequent cloning of other genes involved in N metabolism that co-localize to the already identified QTLs should speed up the release of N efficient crop varieties developed through genetic engineering.

1.8. Genetic Engineering of Plant Nitrogen Metabolism to Increase NUEg

A number of groups have attempted to alter various components of N metabolism using transgenic approaches (Good et al., 2004). Genes including high affinity nitrate transporters (Liua et al., 1999; Fraisier et al., 2000), nitrate reductase (NR) (Djennane et

al., 2002; Lillo et al., 2003), nitrite reductase (NiR) (Crete et al., 1997), glutamate dehydrogenase (Ameziane et al., 2000), glutamine synthetase (GS) (Migge et al., 2000; Habash et al., 2001; Oliveira et al., 2002), glutamate synthase (GOGAT) (Chichkova et al., 2001; Yamaya et al., 2002) and asparagine synthetase (AS) (Lam et al., 2003) have all been over-expressed in a variety of plant species. In the majority of cases, the transgenes were constitutively over-expressed using the cauliflower mosaic virus 35S promoter (CaMV 35S). Generally, alterations to N metabolism, N uptake, and/or biomass are reported with only a few studies indicating alterations in NUEg.

Data from tobacco transgenics however, indicates a possible increase in NUEg. Driving the expression of a bacterial glutamate dehydrogenase with the CaMV 35S promoter, Ameziane et al. (2000) observed a 10% increase in dry weight of greenhouse and field grown plants. Furthermore, constitutive over-expression of either an alfalfa NADH-GOGAT (Chichkova et al., 2001) or an alfalfa cytosolic GS (Fuentes et al., 2001) in tobacco, resulted in a significant increase in shoot biomass and a 100% increase in dry shoot weight respectively. Migge et al. (2000) also showed that leaf specific over-expression of an endogenous chloroplastic GS gene resulted in a 20%-30% increase in tobacco fresh weight, though the difference was attenuated at 12 weeks.

Additional data from experiments using wheat and rice indicate that increases in NUEg are not confined to tobacco. Transgenic wheat plants over-expressing a *Phaseolus vulgaris* cytosolic GS showed significant increases in dry shoot biomass, dry root biomass and grain weight when compared to controls (Habash et al., 2001). Similarly, Yamaya et al. (2002) increased grain weight in rice by 80% via tissue specific over-expression of a rice NADH-GOGOAT gene. These studies and the data from tobacco

transgenics indicate that NUEg can be increased in crop species using a transgenic approach.

1.9. Plant Nitrogen Metabolism

Primary N assimilation involves the uptake of soil NO_3^- into roots, through one of three transport systems: a low affinity transport system, an inducible high affinity transport system, and a constitutive high affinity transport system (Tischner, 2000). Reduction of NO_3^- to NH_4^+ follows through the sequential action of NR and NiR (Oaks, 1994; Vance, 1997). Primary N assimilation also includes the direct uptake of NH_4^+ from the soil via specific ammonium transport systems (Wiren et al., 2000). Fertilizer N is assimilated through primary N assimilation. Photorespiration also generates NH_4^+ (Hopkins, 1999). It is important that a plant re-assimilates this NH_4^+ before it is lost or accumulates to toxic levels within the plant. It is estimated that the amount of photorespiratory NH_4^+ assimilated exceeds that of primary N assimilation by an order of magnitude (Lam et al., 1996; Miflin and Habash, 2002). Finally, NH_4^+ can be released during cellular processes such as protein catabolism (Lam et al., 1996). The recovery of this released NH_4^+ is particularly important during germination and senescence.

Once NH_4^+ is generated through either primary N uptake and reduction, photorespiration, or other cellular processes, the coordinate action of GS and GOGAT reduce the NH_4^+ into a usable, organic form for plant metabolism (Lea and Miflin, 1974) (Figure 1.7). The action of specific isoenzymes of GS and GOGAT, encoded by distinct nuclear genes that direct the expression of their respective isoenzymes to different sub-cellular compartments (Lam et al., 2003), assimilate ammonia generated from specific

cellular processes. Evidence suggests that chloroplastic GS (Kozaki and Takeba, 1996; Migge et al., 2000) and Fd-GOGAT (Coschigano et al., 1998) act to assimilate photorespiratory NH_4^+ where as cytosolic GS (Lam et al., 1996) and NADH-GOGAT (Lancien et al., 2002) assimilate NH_4^+ from primary N metabolism and other cellular processes.

Nitrogen flow in the plant can be continued downstream of the GS/GOGAT cycle through a number of transamination reactions (Ireland, 1997). The action of aspartate aminotransferase (AAT) in particular (Figure 1.7), has been demonstrated to be important in plant N metabolism. Aspartate (Asp) serves as an important N transport molecule in plants (Lea and Miflin, 1980; Ireland, 1997). Furthermore, the pool of aspartate generated by AAT has been shown to be used by distinct AS isoenzymes when photosynthetic rates decline or carbon skeletons become limited (Lam et al., 1998; Miesak and Coruzzi, 2002; Wong et al., 2004). The high N to carbon (C) ratio of asparagine (Asn) provides the plant with a more efficient means to transport N under these circumstances.

1.10. The Role of Aspartate Aminotransferase in Plant Nitrogen Metabolism

Aspartate aminotransferase catalyzes the reversible transamination reaction involving the production of Asp and 2-oxoglutarate from glutamate (Glu) and oxaloacetate. Multiple isoenzymes, encoded by distinct nuclear genes, carry out this reaction in distinct sub-cellular compartments (Wadsworth, 1997; Liepman and Olsen, 2004). In *Arabidopsis*, genes encoding AAT directed to the cytosol, chloroplast/plastid and mitochondria have been identified (Schultz and Coruzzi, 1995; Wilkie et al., 1995;

Wilkie and Warren, 1998). Schultz and Coruzzi (1995) used a RT-PCR based approach to clone four distinct AAT cDNAs in *Arabidopsis*. Based on sequence comparison to known AAT genes *ASP1*, *ASP2* and *ASP4*, and *ASP3* were identified to encode a mitochondrial, cytosolic, and plastid AAT isoenzyme respectively. Wilkie et al. (1995) cloned the fifth AAT gene in *Arabidopsis* from a cDNA library. Using an *in vitro* protein import assay, Wilkie et al. (1995) demonstrated that *ASP5* encodes a chloroplastic AAT isoenzyme. It is hypothesized that the various AAT isoenzymes serve distinct, non-overlapping roles in plant metabolism. Mitochondrial, plastid, and chloroplastic isoenzymes of AAT are proposed to be involved in shuttling reducing equivalents between sub-cellular organelles (Liepman and Olsen, 2004), whereas cytosolic AAT has been shown to serve a non-redundant role in primary N metabolism in *Arabidopsis* (Shultz et al., 1998; Miesak and Coruzzi, 2002). Cytosolic AAT synthesizes Asp during the day, which is then used by the plant to transport N. During the night, when C skeletons are limited, AS uses the pool of Asp created by cytosolic AAT to produce Asn. Asparagine replaces Asp as an N transport molecule during the night because it is deemed to be a more efficient way to transport N by virtue of its high N to C ratio.

1.11. Genetic Engineering of Aspartate Aminotransferase Activity

Three attempts to modify the activity of AAT *in planta* have been reported. Mett et al. (1996) successfully reduced the activity of a nodule specific aspartate aminotransferase (AAT-P_2) in *Lotus corniculatus* by expressing an antisense cDNA copy from a lupin AAT-P_2 with an inducible, tissue specific promoter. The reduction in AAT-P_2 activity was correlated with a reduction in the nodule Asn concentration suggesting

that the nodule Asp formed by AAT-P_2 is the substrate for AS. Mett et al. (1996) did not report any biomass data nor did they attempt to analyze NUEg in the transgenics.

Sentoku et al. (2000) were able to successfully increase the activity of a mitochondrial (mAspAT) and cytosolic (cAspAT) AAT in tobacco plants by over-expressing cDNA copies of either mAspAT or cAspAT from *Panaicum*, using the CaMV 35S promoter. They increased mAspAT and cAspAT activity in leaves 3 and 3.5 times respectively, above that of non-transformed plants. Furthermore, the increased AAT activity was associated with an increase in endogenous phosphoenolpyruvate carboxylase activity implying a link between carbon and nitrogen metabolism. Biomass data was not reported in this study. As such, NUEg can not be assessed in these tobacco transgenics.

Most recently, a *Glycine max* putative chloroplastic AAT was constitutively over-expressed in *Arabidopsis* (Murooka et al., 2002). Analysis of seed amino acid content revealed 23 and 50 fold increases in Asn and glutamine (Gln) concentrations respectively. Both of these amino acids have been implicated as major N transport molecules in plants (Lea and Miflin, 1980; Ireland, 1997). Once again, the authors failed to report any data particular to NUEg. Regardless, the above studies show that genetic engineering can be used to successfully manipulate AAT activity *in planta* and that those manipulations have effects on N and possibly C metabolism.

1.12. *Brassica napus* as a Model Nitrogen Efficient Crop Species

Brassica napus has become a globally important crop species. Since 1961, the harvested area of *B. napus* has increased almost 4 fold to 23.7 million hectares in 2003 (FAO data base, 2004). The increase in harvested area has been accompanied by a

greater than 9 fold increase in global annual seed production. In Canada, yearly seed production and harvested area of *B. napus* has increased 15 and 22 times respectively over the same period (Figure 1.8).

B. napus also lends itself well to Agrobacterium mediated transformation (Moloney et al., 1989). Using this method, Dr. Good's research group has created an N efficient genotype of B. *napus* (Good et al., in prep.). By ectopically over-expressing a barley alanine aminotransferase cDNA in the epidermis of *B. napus* root hairs, transgenic plants showed a 42.3% increase in yield in field experiments at a reduced nitrogenous fertilizer rate (56kg/ha). Furthermore, the transgenic plants, when grown in a hydroponic system, showed increased NO_3^- uptake coupled with an increase in transcript abundance from the *B. napus* high affinity NO_3^- transporters (*BnNrt2.1* and *BnNrt2.2*). These data suggest that NUEg is amendable in *B. napus*.

1.13. Scope of the Thesis

The purpose of this thesis was to test the hypothesis that the targeted over-expression of AAT would result in a similar phenotype to that observed in the transgenic *B. napus* over-expressing an alanine aminotransferase cDNA (section 1.12.). This hypothesis was tested by over-expressing a cytosolic AAT in a tissue specific pattern, using a transgenic approach. Analysis of putative transgenics lines included:

- The identification of transgenic lines containing a single insertion event.
- The creation of transgenic lines homozygous for the transgene from those transgenic lines with a single insertion event.

- A preliminary analysis of nitrogen efficiency in selected transgenic lines homozygous for the transgene.
- An analysis of AAT enzyme activity in selected transgenic lines homozygous for the transgene.
- Confirming the expression of the transgene within selected transgenic lines homozygous for the transgene.

Through this analysis I was able to identify a single independent transgenic line that showed an initial increase in biomass when compared to the control. This line may represent a genotype with improved NUEg.

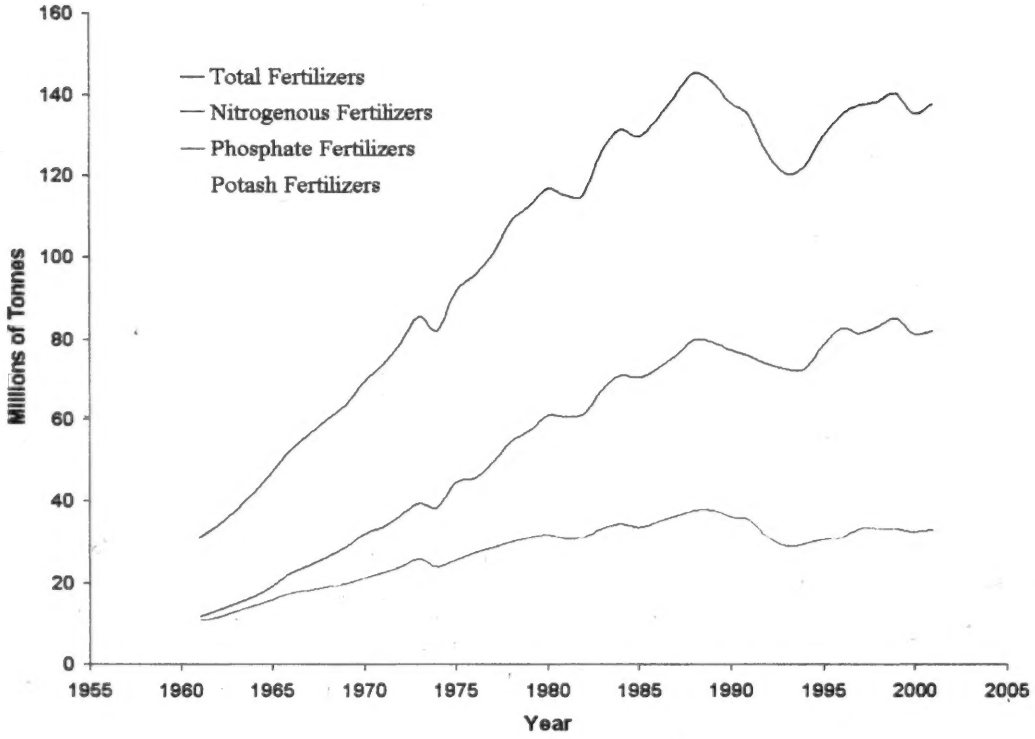

Figure 1.1: World fertilizer consumption from 1961 to 2001. Data obtained from the FAOSTAT-Agriculture data (accessed April 2004).

Table 1.1: Sources and amount of natural and anthropogenic nitrogen fixed globally per year. Value for "Combustion" includes newly fixed nitrogen and remobilized nitrogen stored in the fuel. Data from Vitousek et al., 1997; Socolow, 1999; Gruber, 1997.

Cause	Source	Tg
Natural	Non-Agricultural	130
	Lightning	10
	Sea	30-200
	Total	170-340
Anthropogenic	Industrial (Fertilizer)	95 (80)
	Combustion	20
	Agricultural	40
	Total	155

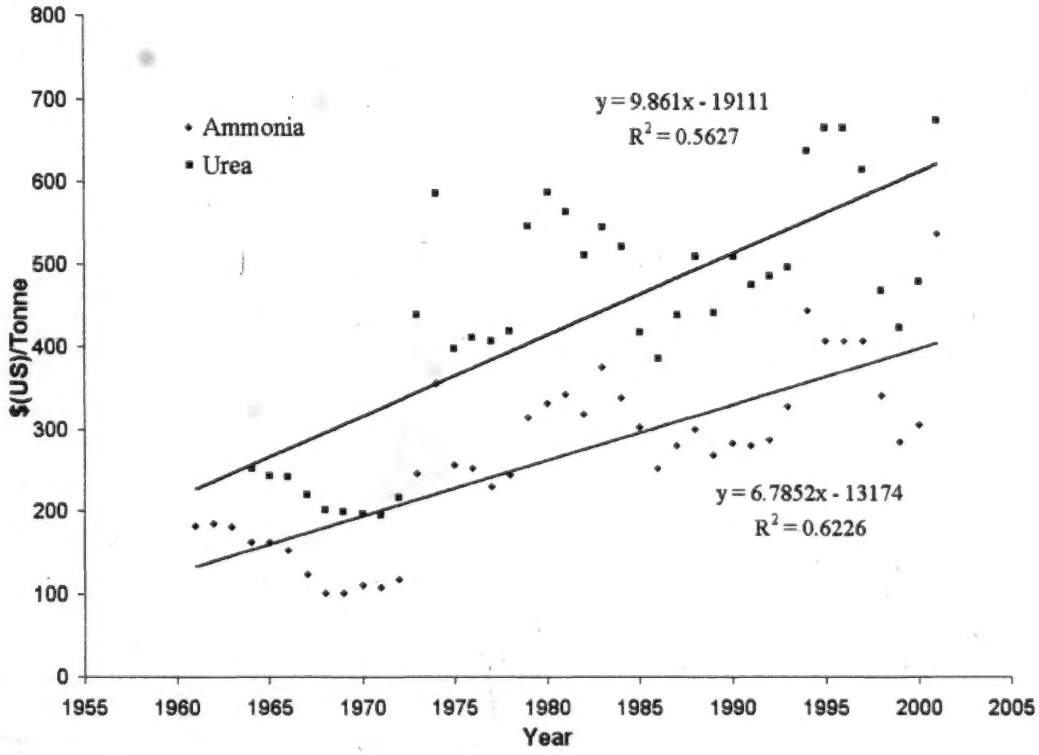

Figure 1.2: Prices paid for selected nitrogenous fertilizers in the US from 1961-2001.

Data obtained from the FAOSTAT-Agriculture data, last accessed April 2004.

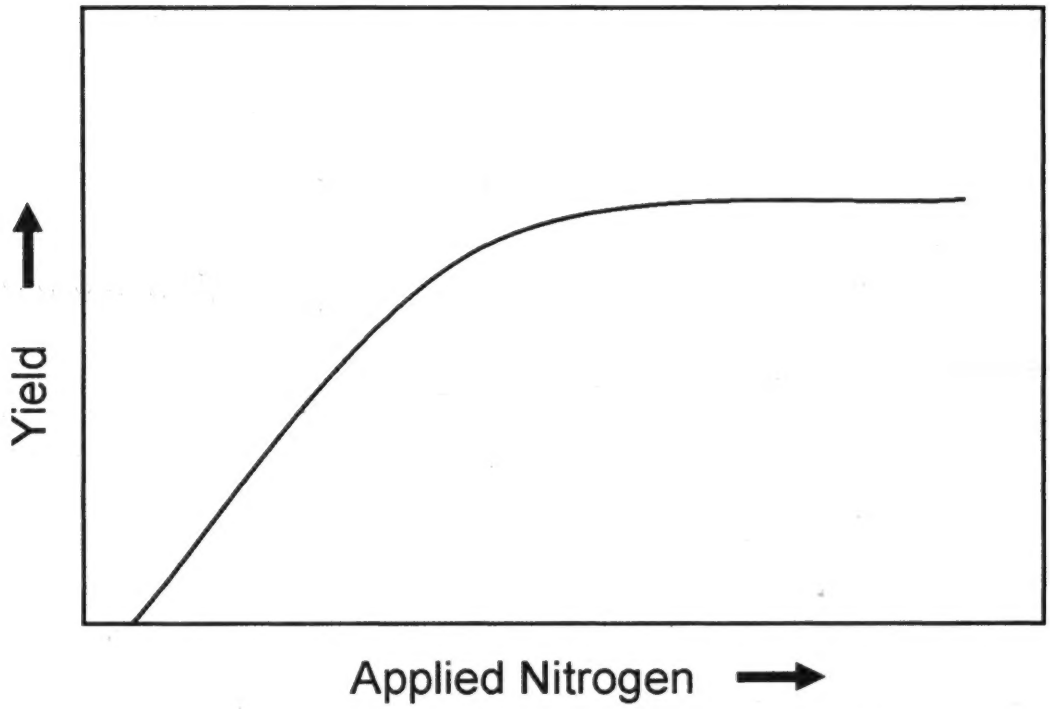

Figure 1.3: Growth response curve for a hypothetical variety of crop species. By plotting the change in yield in response to increasing amounts of applied N the slope of the curve defines NUEg for that cultivar.

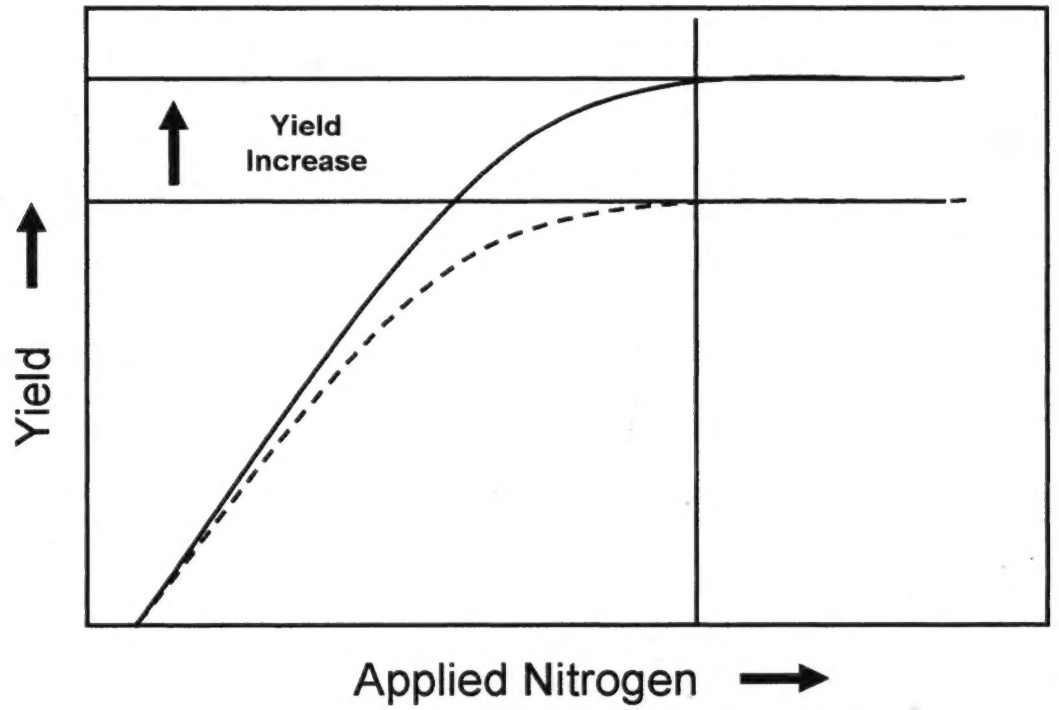

Figure 1.4: Growth response curve of a hypothetical NUEg variety of crop species (solid line) compared to a hypothetical standard variety of the same crop species (dashed line). The increase in NUEg results from an increase in yield with out a proportional increase in applied N.

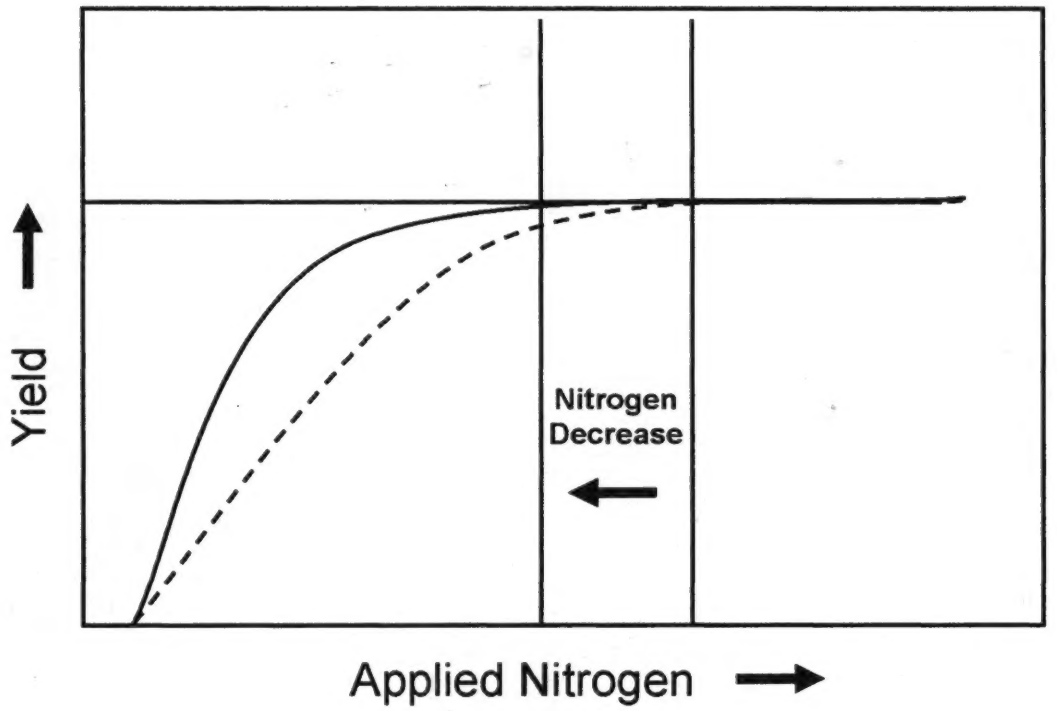

Figure 1.5: Growth response curve of a hypothetical NUEg variety of crop species (solid line) compared to a hypothetical standard variety of the same crop species (dashed line). The increase in NUEg results from a decrease in applied N with out a proportional decrease in yield.

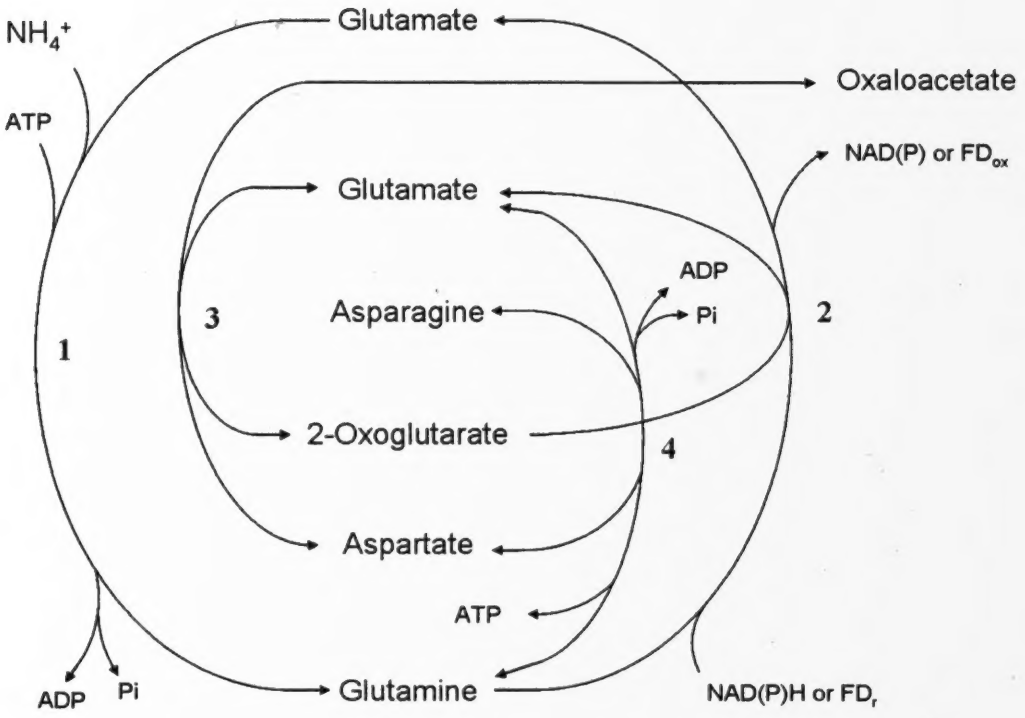

Figure 1.6: Enzymes involved in nitrogen assimilation. Ammonium (NH_4^+) required for nitrogen assimilation is derived from the reduction of nitrate taken-up by roots, produced by photorespiration and other cellular reaction, or directly taken-up by roots. The coordinated actions of glutamine synthetase (1) and glutamate synthase (2) reduce the bulk of NH_4^+ into usable, organic metabolites. Aspartate aminotransferase (3) and asparagine synthetase (4) are involved in the production of the nitrogen transport molecules aspartate and asparagine respectively.

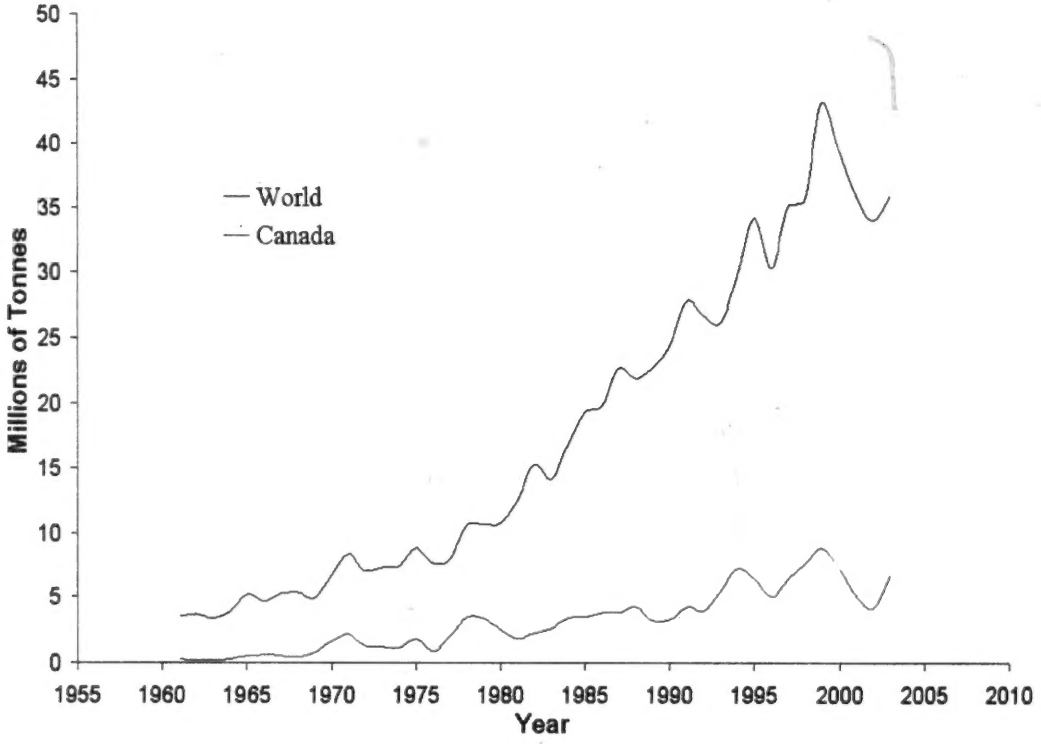

Figure 1.7: Global and Canadian rapeseed production. Data obtained from the FAOSTAT-Agriculture data, last accessed April 2004.

2: Methods

2.1. Construction of the Binary Vector

A 1270 bp region of a cytosolic aspartate aminotransferase (*AAT-1*) cDNA cloned from *Medicago sativa* by Udvardi and Kahn (1991) was amplified using primers P10 (5'-CCGCTCGAGATGTCTGATTCCGTCTTCGCTCA -3') and P11 (5'-CCGCTCGAGTCACGGGGATGAATTGATAA-3'). The primers were designed to introduce Xho I restriction endonuclease cut sites at the 5' and 3' end of the PCR fragment. Klenow, dTTP and dCTP were used to partially fill in the Xho I sticky ends, creating Bam H1 compatible ends on the 1270 bp AAT-1 PCR product. This fragment was cloned into the Bam H1 restriction site within the poly linker of p25 (gift from Maurice Moloney, University of Calgary). p25 was derived from a pUC19 vector by inserting a duplicated CaMV 35S promoter (Kay et al., 1987) and NOS terminator, linked by a Bam HI, Xba I, and Pvu I polylinker, between the Kpn I and Pst I restriction cut sites of pUC19. Only clones where AAT-1 had inserted in the sense orientation were carried forward. The duplicated CaMV 35S promoter of p25 was switched out for a 300bp fragment of the 5' promoter region of *btg-26* (Stroeher et al., 1995) prior to the insertion of the AAT-1 PCR product into p25. The Kpn I/Pst I restriction fragment containing the *btg-26* promoter, the AAT-1 PCR fragment and a NOS terminator, derived from the original p25 vector, was subcloned between the Kpn I and Pst I restriction endonuclease sites of pCGN1547 (McBride and Summerfelt, 1990) to create the binary vector p26gAspATNS47 (Figure 2.1). This work was completed between 1998 and 2000 by Natalie Labbé, a research technician in the lab of Dr. Allen Good.

2.2. *Agrobacterium* Mediated Transformation

p26gAspATNS47 was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation following the protocol outlined in the BTX Electro Cell Manipulator ECM 600 manual. This work was completed between 1998 and 2000 by Natalie Labbé under the supervision of Dr. Allen Good. The resulting transformed *Agrobacterium* was used to transform a double haploid line of *Brassica napus* cv Westar (N-o-1) following the protocol of Moloney et al.(1989). Putative T_0 transformants were screened by isolating genomic DNA from leaf tissue (as described in section 2.5.1.) and the presence or absence of the transgene was confirmed by amplifying a portion of the selectable marker, the NPT II gene contained within the borders of the transgene, by PCR (section 2.8.1.) using primers P18 and P19 (section 2.7.). Thirty-nine putative transgenic lines representing presumed independent insertions were found to contain a portion of the transgene. The *Agrobacterium* mediated transformation of N-o-1 was completed by Mary DePauw, a research technician working in the lab of Dr. Allen Good, between 2000 and 2001. Putative transformants were selfed to produce a T_1 generation.

2.3. Nomenclature

All experimental lines are given an identification number (ID#) that provides information as to the generation and descent of that line. All ID#s in this experiment begin with "6W". The number "6" denotes the transformation experiment and "W" indicates the variety (ie. "Westar") of *B. napus* used as the background for the transformation. The number immediately following "W" refers to individual T_0 shoots from separate and distinct calli. The subsequent letter differentiates separate T_0 shoots

originating from the same callus. These shoots may or may not represent independent insertions of the transgene. The T_1 generation of plants is designated by the symbol "#" followed by a number between 1 and 20. To designate the T_2 generation the ID#s end with a period followed by a number between 1 and 4 or the word "mix", which indicates that the T_3 seed was bulked. For example 6W-13c#18.3 identifies this plant as a T_3 individual derived from the 3rd T_2 plant descended from the 18th T_1 plant. The 18th T_1 plant descended from the "c" shoot of the 13th transgenic callus derived by transforming *B. napus*, N-o-1 during the 6th transformation experiment. Likewise, 6W-14b is a T_0 plant derived from the "b" shoot of the 14th transgenic callus from the same transformation.

2.4.1. Plant Growth Conditions: Increases

To produce subsequent generations, one seed was placed one to two centimetres below the pre-wetted substrate (Terra-Lite 2000, Metro-Mix 220 growing media) surface in pot 15 cm in diameter. Plants were grown in growth chambers at 20°C during the day and 16°C during the night. Relative humidity was set at 60%. Days were 16 hours long with a light intensity of 240 μmol photon/m^2·s at bench height. All plants were watered three times per week and fertilized with an all purpose fertilizer (Plant Products, 20-20-20 Plant-Prod) as recommended by the manufacturer. Prior to anthesis, individual plants were covered with clear, plastic pollination bags to ensure self fertilization. As the plants reached maturity, water and fertilizer were slowly reduced and eventually eliminated, allowing the seeds to be collected once the plants dried out completely.

2.4.2. Plant Growth Conditions: Biomass Experiment

Six inch pots were filled with vermiculite, soaked with tap water and two or three seeds were placed one to two centimeters below the surface. Transgenic seeds were from the T_2 generation. Potted plants were placed in growth chambers for five or six weeks with growth conditions similar to those described in section 2.4.1, but with the following changes. Water was supplied to the plants as needed. Beginning the second or third week of the experiment, each pot received 200 μl of 20 mM FeEDTA once a week, six millilitres of fertilizer solution (152 mM $MgSO_4 \cdot 7H_2O$, 14.53 mM $K_2HPO_4 \cdot 2H_2O$, 274.87 mM KH_2PO_4, 694 nM H_3BO_3, 104 nM $MnSO_4 \cdot 4H_2O$, 11.5 nM $ZnSO_4 \cdot 7H_2O$, 16.5 nM $CuSO_4 \cdot 5H_2O$, 1.25 nM $Na_2MOO_4 \cdot 2H_2O$) on Monday and Wednesday and a nine millilitre aliquot of the same on Friday. The fertilizer solution also contained urea at one of two concentrations. High N fertilizer contained 356 mM urea and low N fertilizer contained 120 mM urea resulting in a total of 60 mmol of N for each plant fertilized with the high N fertilizer and 20 mmol of N for each plant given the low N fertilizer (Table 2.1). Plants were culled, prior to the start of the fertilizer and FeEDTA treatments, so that each pot contained one plant of similar size. Trays under each pot prevented the loss of nutrient solutions through the bottom of the pot. Fourteen plants from a single line were equally divided and randomly assigned to either the high or low N fertilizer treatment. A single growth chamber was sufficient to test a maximum of nine separate lines at both N concentrations. Plants surviving the treatment were harvested at the end of the five or six week period by separating the root and shoot portion of each plant and recording their respective fresh weights. Prior to recording the root weight, the vermiculite was washed off and the roots were blotted dry. Dry root and shoot weights were recorded after drying

the tissue at 65°C for three to four days. The largest and smallest plants, determined by dry biomass, from each line respectively, were removed from further analysis in both fertilizer treatments.

2.4.3. Plant Growth Conditions: Hydroponics

Ten to twelve seeds from the bulked T_3 generation of individual transgenic lines were surface sterilized in a 20% bleach solution for 15-20 minutes. Seeds were then rinsed three times with sterile water and plated out onto germination medium (1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM K_2SO_4, 2 mM $CaCl_2$, 0.05 mM $K_2HPO_4 \cdot 2H_2O$, 0.95 mM KH_2PO_4, 20.82 nM H_3BO_3, 3.12 nM $MnSO_4 \cdot 4H_2O$, 0.345 nM $ZnSO_4 \cdot 7H_2O$, 0.495 nM $CuSO_4 \cdot 5H_2O$, 0.045 nM $Na_2MOO_4 \cdot 2H_2O$, 10 nM FeEDTA, 1.25 g/ 500 ml Phytagar (Gibco, Cat. No. 10675-023) at pH 5.2-5.5) under sterile conditions. After four to five days, seedlings were transferred to previously sterilized, 20 l aquaria with the glass blacked out. The tanks contained 18 l of a nutrient solution (2 mM $NaNO_3$, 0.25mM NH_4NO_3, 1 mM $MgSO_4 \cdot 7H_2O$, 0.5 mM K_2SO_4, 2 mM $CaCl_2$, 0.05 mM $K_2HPO_4 \cdot 2H_2O$, 0.95 mM KH_2PO_4, 41.64 nM H_3BO_3, 6.24 nM $MnSO_4 \cdot 4H_2O$, 0.69 nM $ZnSO_4 \cdot 7H_2O$, 0.99 nM $CuSO_4 \cdot 5H_2O$, 0.09 nM $Na_2MOO_4 \cdot 2H_2O$, 20 nM FeEDTA at pH 5.2-5.5) based on Long Ashton nutrient solution (Hewitt, 1966). pH was monitored daily and maintained by the addition of appropriate amounts of 1 M HCl or 1 M NaOH. Gentle aeration was provided to supply O_2 and prevent stratification of nutrients. Growth conditions were as described in section 2.4.1. Three weeks post germination, at the fourth or fifth leaf stage, the newest portion of the roots and a sample of the fourth leaf

(where possible) was flash frozen and stored at -80°C for further analysis. Harvesting of plant tissue occurred between 11:00AM and 2:00PM.

Osmotic stress is known to induce expression of *bgt-26* (Stroeher et al., 1995). As such, the addition of 100 mM NaCl was used to induce transcription from the transgene. Aquaria received 25 mM NaCl five days prior to harvest, another 25 mM NaCl four days prior to harvest and a final dose of 50 mM NaCl two days prior to harvest, for a total of 100 mM NaCl.

For aspartate aminotransferase enzyme assays, a single aquarium contained five plants from one transgenic line and five plants from the corresponding negative sibling. Two to three days after transferring the seedling to the aquaria, two plants from each line were culled leaving three plants per line. Two aquaria were set up for each line. One aquarium was treated with 100 mM NaCl (as above) and the other was not. For the analysis of transgene expression, three aquaria where set up as outlined above. A single plant from lines 6W-4a#5.mix, 6W-8c#11.mix, 6W-9a#4.mix, 6W-11b#2.mix, 6W-13c#18.mix, 6W-12d#19.mix, 6W-14b#9.mix, 6W-14b#15.mix and N-o-1 were randomly assigned a separate location in each of the three tanks.

2.5.1. Nucleic Acid Isolation: Genomic DNA for PCR

Genomic DNA was isolated following the protocols outlined by Edwards et al. (1991). Leaf discs or five to six day old whole seedlings were ground in individual micro centrifuge tubes and 400 µl of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS) was added. After centrifuging the tubes, 300 µl of the supernatant was removed and a similar volume of isopropanol added to the

supernatant. Following a brief incubation at room temperature, the samples were centrifuged, the supernatant was discarded and the DNA was re-suspended in 200 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

2.5.2. Nucleic Acid Isolation: RNA

Qiagen's RNeasy Plant mini kit (Cat. No. 74904) was used to isolate total RNA from a 100 mg of plant tissue harvested as per section 2.4.3. The manufacturer's directions were followed for all steps. The final elution was done using two 30 µl volumes of RNase free water. RNA was stored at -20°C.

2.6. Protein Isolation: Total Soluble Protein

Plant tissue harvested as per section 2.4.3. was ground in an ice cold mortar containing extraction buffer (0.5 mM EDTA, 0.1 mM dithiothreitol, 10 mM cysteine, 0.1 mM phenylmethanesulfonyl fluoride, 0.005 mM leupeptin, 100 mM Tris-HCl pH 7.8) in a 3:1 ratio of buffer to tissue (µl:µg) with a pinch of acid purified sand and poly(vinylpolypyrrolidone). The resulting slurry was centrifuged to clear debris and the supernatant stored on ice.

2.7. Primers

Primer pair P18 and P19 were previously designed to amplify a fragment from the NPT II gene. All remaining primers were designed using the web based application Primer3 (Rozen and Skaletsky, 2000). The MSASPAT primer pair amplify the 3' region of the AAT-1 cDNA contained within the transgene whereas the BNACTIN primer pair

amplify a portion of an endogenous *B. napus* actin mRNA (Genebank Accession# AF111812). For primer sequences and product sizes refer to Table 2.2.

2.8. PCR

All PCR reactions were carried out on either the GeneAmp PCR System 9600 (Perkin Elmer) or the GeneAmp PCR System 9700 (PE Applied Biosystems). Cycling conditions included an initial five minute denaturation at 95°C followed by 30 cycles of 94°C for one minute, 60°C for two minutes and 72°C for one minute. A final 10 minute elongation period at 72°C was included and the samples where then held at 4°C. The reactions were carried out in a 1X PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.0, 0.01% gelatin). dNTPs were supplied at a concentration of 0.4 mM for each dNTP, $MgCl_2$ was supplied at a concentration of 2.5 mM and 0.5 µl or 1.0 µl of an in-house Taq DNA polymerase was used for 25 µl and 50 µl PCR reactions respectively. Two and a half or five microlitres of DNA isolated as per section 2.5.1. was used as the template for 25 µl and 50 µl PCR reactions respectively.

2.9. DNA Gel Electrophoresis

Six times (6X) loading dye (0.25% bromophenol blue and 40% sucrose) was diluted to 1X in DNA samples to be size fractioned. The resulting samples were electrophoresed through a 1% agarose gel in 1X TAE running buffer at 70 mV to 100 mV (depending on the dimensions of the gel) until the dye line had progressed approximately half way down the gel. Gels images were captured and visualized using the AlphaImager

2200 Documentation and Analysis System device (Alpha Innotech Corporation) and the images stored as TIF files.

2.10. Aspartate Aminotransferase Enzyme Assay

Aspartate aminotransferase activity was determined spectrophotometrically essentially as described by Rej and Horder (1983). The protocol was modified to reduce the assay volume from 2.4 ml to 150 μl allowing for high through put analysis using a microplate spectrophotometer (SpectraMAX Plus, Molecular Devices). Ten microlitres of a diluted aliquot of the total protein isolated as per section 2.6. was added to 120 μl of assay buffer (240 mM aspartate, 100 mM Tris-HCl pH 7.8, 0.11 mM pyridoxyl 5-phosphate, 0.16 mM NADH, 1.0 U/ml malate dehydrogenase (Sigma, Cat. No. M2634), 0.455 U/ml lactate dehydrogenase (Sigma, Cat. No. L2518)). The change in A_{339} was measured for five minutes to determine background activity levels. Once the background activity was minimal, 20 μl of 2-oxoglutarate was added and the change in A_{339} was again measured for five minutes. Each sample consisted of three technical replicates and the average of these replicates was used to determine the activity of a sample. The background activity of a sample was found following the same procedure, but 2-oxoglutarate was not added.

2.11. Quantification of Total Soluble Protein

The concentration of total soluble protein was measured using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, Cat. No. 500-0006). Samples extracted in section 2.6. were assayed according to the manufacturer's instructions using

a microplate spectrophotometer (SectrMAX Plus, Molecular Devices). Bovine serum albumin was used as the protein standard. All samples were assayed in triplicate and the average for each sample recorded.

2.12. RT-PCR

Contaminating DNA in total RNA extracted from plant tissue as per section 2.5.2. was removed using Ambion's DNA-Free Kit (Ambion, Cat No. 1906) following the recommended procedure. Two and a half microlitres of the resulting total RNA suspension was incubated for 1.5 hours with 50 U of SuperScript II Reverse Transcriptase (Invitrogen, Cat. No. 18064-022) and gene-specific primers, following the manufacturer's directions. Primers MSASPAT 3' or BNACTIN 3' initiated first strand cDNA synthesis from AAT-1, contained within the transgene, and *B. napus* actin mRNA, Genebank Accession# AF111812, respectively. Twenty-five microlitre PCR reactions (section 2.8.) were used to amplify first stand cDNA. A half unit of Platinum Taq DNA Polymerase (Invitrogen, Cat. No. 10966-018) was substituted for the in-house Taq and five microlitres of the reverse transcriptase reaction supplied as the template. Primer pair MSASPAT 5' and MSASPAT 3' amplified a portion of the AAT-1 first stand cDNA. Primer pair BNACTIN 5' and BNACTIN 3' amplified a portion of the actin first stand cDNA. PCR reactions were analyzed by DNA gel electrophoresis as described in section 2.9. A second 2.5 μl aliquot of total RNA from each sample was used to control for the presence of genomic DNA contamination by subjecting it to a similar procedure described above but withholding the reverse transcriptase enzyme during the first stand cDNA synthesis.

2.13. Biossay for Neomycin Phosphotransferase II Expression

Approximately 12 seeds from individual lines where surface sterilized as described in section 2.4.3. and plated out on half strength Murashige and Skoog basal medium (Sigma, Cat. No. M5519) containing one percent sucrose, 2.5 g/l of phytagar (Gibco, Cat. No. 10675-023) and 150 μg/ml kanamycin in 60 mm X 15 mm petri dishes. Lids were left off and the plates were placed inside sterilized Magenta jars. Seeds were allowed to germinate for ten days in a growth chamber. Environmental conditions were as described in section 2.4.1. Individual cotyledons were scored as either completely bleached, partially bleached or not bleached. The experiment was repeated a second time using approximately 18 seeds per line and plates were incubated for 14 days. Both experiments utilized T_3 plants.

2.14. Statistical Analysis

Dry root and shoot biomass was compared between transgenic lines in experiment one and two using SPSS 11.5 statistical soft wear for windows. Statistically significant differences between both dry root biomass and dry shoot biomass of the various transgenic lines treated with the high and low N fertilizer treatments respectively (section 2.4.2.) were detected using an ANOVA. Transgenic lines with statistically different mean dry root biomass and mean dry shoot biomass were determined in the high and low nitrogen fertilizer treatments respectively using Tukey's multiple comparisons test. Statistical significance was established at an α value of 0,05.

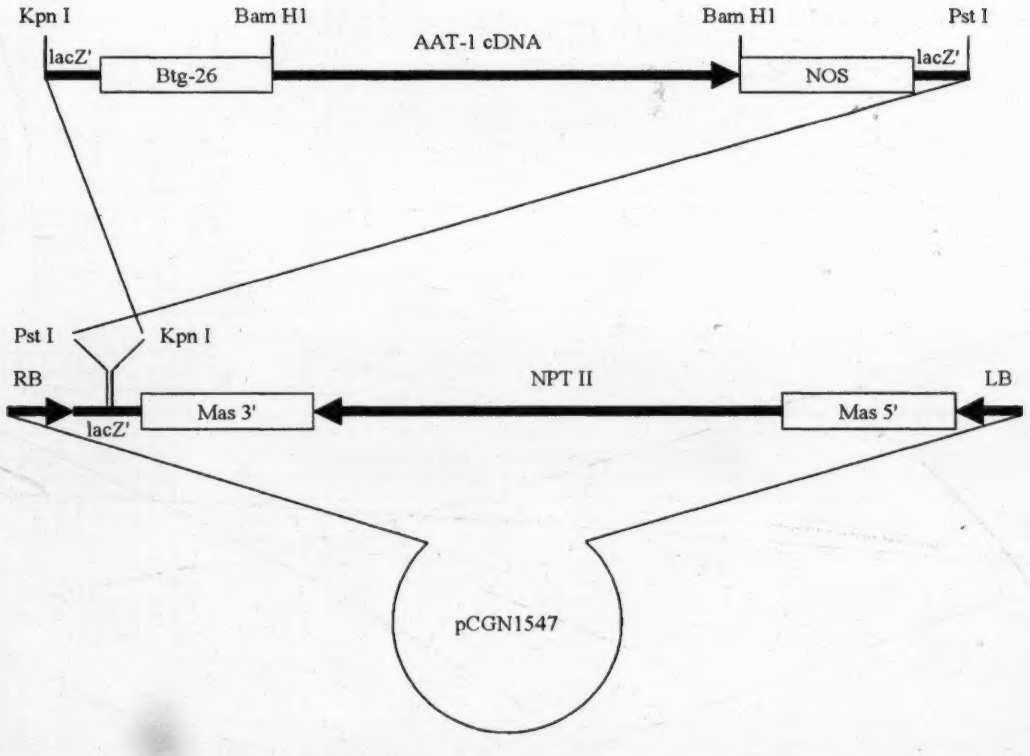

Figure 2.1: Binary vector p26gAspATNS47. The region between the left and right border of the binary vector pCGN1547 (McBride and Summerfelt, 1990) has been magnified. The map is not to scale.

Table 2.1: Nitrogen fertilizer treatments. Plants were treated with the appropriate urea based fertilizer three times weekly for a total of four weeks. Each pot received six millilitres of fertilizer Monday and Wednesday and nine millilitres on Friday.

	Concentration of fertilizer applied per treatment (mM)	Total fertilizer or nitrogen applied per plant (mmoles)		Equivalent rate of nitrogenous fertilizer (kg of N/ha)
		Urea	Nitrogen	
Low N fertilizer	120	10	20	165
High N fertilizer	356	30	60	495

Table 2.2: List of primers used.

Primer Name	Sequence 5' – 3'	Target	Product Size (bp)
P18	CGCTCAGAAGAACTCGTCAAGAA	Portion of the NPT II gene contained within the transgene	656
P19	TTTGTCAAGACCGAC TGTCC		
MSASPAT 5'	AACATGGGTCTTTATGGTGAACGTG	Portion of the AAT-1 cDNA contained within the transgene	498
MSASPAT 3'	TCACGGGGATGAATTGATAACAAAC		
BNACTIN 5'	GGTCGTCCTAGGCACACTGG	*B. napus* actin mRNA, Genebank Accession # AF111812	586
BNACTIN 3'	TAGCCGTCTCCAGCTCTTGC		

3. Results

3.1. Screening of Putative Transgenics

Thirty-nine putative T_0 transgenic lines, representing presumed independent insertion events, were selfed to advance the lines to the next generation. All of the 39 independent transformants were previously shown to contain a portion of the selectable marker contained within the transgene as determined by PCR (Mary DePauw, unpublished data). To confirm these results, an identical portion of the transgene was again amplified in individual T_1 plants from each line using primers P18 and P19. Only two lines, 6W-9b and 6W-17, did not contain this portion of the transgene in any of the 20 T_1 plants examined respectively (data not shown). 6W-9b and 6W-17 were excluded from further study.

It is known that *Agrobacterium* mediated transformation generates a number of possible T-DNA integration patterns (Tinland, 1996). As such, the above 37 independently transformed plants could contain either 1) a single transgene at a single locus, 2) multiple transgenes at a single locus, 3) a single transgene at multiple loci or, 4) multiple transgenes at multiple loci. In order to reduce the number of transgenic lines to characterize, a screening system was set up that could identify transgenic lines containing a single or multiple transgene at a single locus (single insertion event).

The PCR results above were used to determine the presumed number of insertion events per line. As an example, of the 20 T_1 plants from line 6W-14b screened, 13 contained an amplifiable portion of the selectable marker (positive plants) and seven plants did not contain an amplifiable portion of the same (negative plants) (Figure 3.1).

This ratio of positive to negative plants was compared to the expected ratio of 15:5 for a single insertion event using the chi-square statistic. As $\chi^2_{0.05,1} = 3.841$ and the calculated χ^2 value is 1.067 for a single insertion event, it can be predicted that line 6W-14b contains a single insertion event.

Using the chi-square test, the number of insertion events was determined in 29 of the remaining 37 independent transgenic lines (Table 3.1). Of the 29 lines, 17 lines had a single locus of insertion and 12 lines had multiple insertion events. Only 11 of these 17 transgenic lines containing a single insertion event were advanced to the T_2 generation. These T_1 transgenic lines had the greatest vigor and the most seed (assessed visually). Care was taken to advance lines representing the greatest number of putative independent insertion events.

The number of insertion events contained within lines 6W-4d and 6W-15c could not be determined because their respective T_0 plants did not produce viable seed. The observed T_1 ratio for line 6W-10d was 10:10; a ratio that does not conform to a single, double, or multiple insertion events. Consequently, a valid estimate of the number of insertion events contained within the genome of line 6W-10d could not be made. As a result, lines 6W-4d, 6W-15c, and 6W-10d were excluded from further analysis. Furthermore, T_0 plants from lines 6W-4a, 6W-4b, 6W-14a, 6W-15a, and 6W-15b did not produce enough seed to generate the required 20 T_1 plants needed for screening. The number of insertion events in each of these lines was not determined. However, the T_1 plants that these lines did produce, one for lines 6W-4b, 6W-14a, 6W-15a, and 6W-15b and three for line 6W-4a, were all positive. These lines were also advanced to the T_2 generation.

In total, 16 independent transgenic lines were advanced to the T_2 generation. The T_2 generation was used to identify T_1 lines that were homozygous for the transgene. The screening procedure consisted of randomly choosing six T_1 plants from each of the 16 independent transgenic lines and advancing them to the T_2 generation. The T_2 progeny from these six plants were screened using PCR to identify those individuals that contained a portion of the transgene using primers designed to amplify a 3' region of the aspartate aminotransferase (AAT) cDNA contained within the T-DNA. If the T_1 plant was homozygous for the transgene, all T_2 progeny should contain an amplifiable portion of the AAT transgene. If the T_1 plant is hemizygous for the transgene, then it is predicted that only three quarters of the T_2 progeny will contain an amplifiable portion of the AAT transgene.

Line 6W-14b can be used to illustrate the procedure employed. T_1 plants 1, 3, 9, 11, 13, and 19 were randomly chosen from the 13 6W-14b T_1 plants containing an amplifiable portion of the selectable marker (Figure 3.1) and advanced to the T_2 generation. PCR amplification of the AAT transgene in twelve T_2 plants germinated from lines 6W-14b#1, 9, 11, and 13 respectively revealed that 6W-14b#9 was homozygous for the transgene (Figure 3.2). Lines 6W-14b#3 and 6W-14b#19 were not screened.

A line homozygous for the transgene was identified for 13 of the 16 T_1 plants advanced to the T_2 generation (Table 3.2). A homozygous line representing the insertion events that occurred in lines 6W-4b and 6W-15b could not be identified because both 6W-4b#1 and 6W-15b#1 failed to produce adequate seed for screening. 6W-4b#1 and 6W-15b#1 were removed from further analysis. Similarly, line 6W-15a#1 was removed

from further analysis as it was determined to be hemizygous for the transgene. All 13 lines homozygous for the transgene were advanced to the T_3 generation with the purpose of increasing the amount of seed available for characterizing the independent transgenic lines.

3.2. Negative Controls

In order to characterize transgenic plants and demonstrate that an observed genetic, physiological, metabolic, or phenotypic effect is the sole result of the transgene, an appropriate control plant must be used to exclude other variables as the cause of any observed effect. Other variables may include the initial germplasm used for transformation, the tissue culture procedure (somaclonal variation) (Jain, 2001; Bhat and Srinivasan, 2002), or the insertion event (Tinland, 1996; Theuns et al., 2002, Wilson et al., 2004). In this thesis, negative siblings were used as the control plants because they were easy to identify during the screening for single insertion events (section 3.1.). Furthermore, negative siblings can control for potential variation caused by both the initial germplasm and somaclonal variation. A single negative sibling was identified for 10 of the 13 T_2 lines advanced to the T_3 generation (Table 3.3). These T_1 negative siblings were advanced to the T_3 generation. Negative siblings for lines 6W-4a and 6W-14a do not exist because a negative plant did not segregate out in the T_1 progeny in either of the lines. Likewise, a negative sibling does not exist for line 6W-7a. Viable seed was not produced from the 6W-7a#7 negative plant.

3.3. Neomycin Phosphotransferase II Bioassay

Neomycin phosphotransferase II (NPT II) activity was assayed in seven of the 13 lines advanced to the T_3 generation to verify that the selection of homozygous lines and their corresponding negative sibling lines was successful. The bioassay consisted of germinating T_3 seeds on half strength Murashige and Skoog basal medium containing 150 μg/ml kanamycin. Neomycin phosphotransferase II activity was confirmed in the T_3 progeny by observing seedlings resistant to kanamycin (Kan^R; seedlings with dark green cotyledons after two weeks growth on the kanamycin containing medium). Transgenic lines derived from a T_2 parent homozygous for the transgene should produce 100% Kan^R seedlings and the cotyledons of seedlings from negative siblings should all appear white or various shades of purple after two weeks of growth on the kanamycin containing medium (Kan^S) (see Figure 3.3 for an example).

Of the transgenic lines analyzed, T_3 seedlings from lines 6W-8c#11, 6W-13c#18, and 6W-14b#9 were all 100% Kan^R confirming that these lines are homozygous for the transgene (Table 3.3). T_3 Seedlings from the remaining lines, 6W-9a#4, 6W-12d#16, 6W-4a#5, and 6W-11b#2, were found to be 70%, 82%, 90%, and 94% Kan^R respectively. This result suggests that these lines are hemizygous rather than homozygous for the transgene, or that the kanamycin assay was not reliable.

The results for the bioassay with the negative siblings suggest that the results from the PCR screen used to identify the negative siblings were not reliable (Table 3.3). Only lines 6W-9a#4, 6W-11b#2, 6W-12d#19, and 6W-13c#18 contained no Kan^R T_3 seedlings whereas lines 6W-8c#10 and 6W-14b#9 had 25% and 16% Kan^R seedlings respectively.

Curiously, it was observed that transgenic lines containing a few T_3 Kan^S seedlings had corresponding negative sibling lines with no Kan^R T_3 seedlings whereas transgenic lines containing no T_3 Kan^S seedlings had corresponding negative siblings with a few Kan^R T_3 seedlings (Table 3.3). The only exception to this observation was line 6W-13c#18 and its negative sibling, line 6W-13c#12. One hundred percent of the T_3 seedlings from 6W-13c#18 were Kan^R whereas none of the T_3 negative sibling seedlings were Kan^R.

3.4. Integration of Full-Length T-DNA

A number of lines of evidence were used to confirm that the transgenic lines not only contained a portion of the transgene but that at least one copy of the transgene was full length. Initial PCR screening to identify transgenic lines containing a single insertion event confirmed the presence of the central region of the NPT II coding region in 37 of the original 39 putative transgenic lines (section 3.1.). Amplification of a 498 bp region at the 3' end of the AAT cDNA contained between the right and left borders of the T-DNA confirmed the presence of this sequence in 13 of the 37 transgenic lines containing the central portion of the NPT II (section 3.1.). The presence of this band implies that a full length AAT cDNA is contained within these 13 transgenics because sequences at or near the right border are highly conserved (Tinland, 1996) (Figure 3.4). The AAT cDNA contained within the transgene should be capable of producing a full length transcript.

The presence of the central portion of NPT II does not however, confirm that NPT II can produce a full length transcript. Deletions to T-DNA sequences at or near the left border are common (Tinland, 1996; Theuns et al., 2002) and NPT II is situated adjacent

to this border (Figure 3.4). A deletion to the promoter driving NPT II gene expression and/or the NPT II sequence upstream of the 5' primer binding site would go unnoticed by relying only on the amplification of the central portion of NPT II. The bioassay confirmed that NPT II is expressed in seven of the 13 lines that contain an amplifiable portion of AAT cDNA and NPT II. If there were deletions to the mas 5' promoter and/or the 5' region of NPT II, none of the T_3 seedlings from the transgenic line carrying the deleted T-DNA would be kan^R. The observation that all seven transgenic lines contained some kan^R seedlings and that they all have an amplifiable portion of the AAT cDNA supports the conclusion that the seven transgenic lines screened for kanamycin resistance contain a copy of a full length T-DNA.

3.5. Phenotypic Evaluation of Transgenic Lines

Growth chamber experiments were used to assess the effect the cytosolic AAT transgene had on the growth phenotype of the transgenic lines. Two to three seeds from each line were randomly assigned to seven different pots treated with a total of 20 mmol of nitrogen (N) and another two to three seeds from the same line were assigned to seven different pots treated with a total of 60 mmol of N. Vermiculite was used for the substrate because it lacks mineral nutrients, making the urea supplied the only source of N. Seeds were germinated in the vermiculite and grown for two weeks at which time a fertilizer treatment was initiated. Prior to the commencement of fertilization, seedlings were culled to one plant of equal size per pot. Water was supplied as needed up until the plants were harvested. Harvesting consisted of separating the root and shoot portions of each plant, removing the vermiculite attached to the roots, and drying out the roots and

shoots in an oven. Dry root and shoot weights were recorded for each plant that survived the experiment and the largest and smallest plants from each line, in both treatments, were removed from further analysis.

It should be noted that the control line for the growth chamber experiment was the background *B. napus* genotype used in the original transformation (N-o-1). The negative sibling for each line could not be used because not all lines tested had a negative sibling (see section 3.2.) and there was not adequate space in the growth chambers to efficiently screen a number of transgenic lines and their respective negative siblings simultaneously.

Of the six transgenic lines initially screened, only 6W-14b#9 had significantly increased root and shoot biomass (Figure 3.5 and 3.6). Mean dry shoot weight of these plants fertilized with the high and the low N fertilizers respectively were 1.6 and 1.7 times greater than control plants and mean dry root weight of plants treated with the high N fertilizer was 2.9 times larger than the control plants. A non-significant increase of 2.1 times over control plants was also observed for mean dry root weight of plants from line 6W-14b#9 fertilized with the low N fertilizer.

The remaining five lines screened either had similar or reduced mean dry shoot and root biomass when compared to the control line. Results for 6W-4a#5, 6W-8c#11, 6W-10a#19, and 6W-12d#19 were not significantly different than the controls treated with either the high or low N fertilizers (Figure 3.5 and 3.6). These lines were considered to be comparable to the control line with respect to growth. Plants from line 6W-9a#4 had significantly smaller shoots in both the high and low N treatments and significantly smaller roots in the high N treatment. Mean dry root weight of plants fertilized with the low N fertilizer however, were only found to have a non-significant reduction in mean

dry root weight compared to the control plants. Plants from this line also exhibited an aberrant phenotype (Figure 3.7). Individuals were stunted, had poor seed set, and leaf morphology was abnormal when compared to N-o-1 and its negative sibling.

A second growth chamber experiment was set up in order to replicate the biomass increases in line 6W-14b#9 observed in the first growth chamber experiment. Lines 6W-4a#5 and 6W-8c#11 were also included because they were deemed to represent genotypes with possible increases in root and shoot biomass (Figures 3.5 and 3.6). The experimental design was identical to that described above for the first growth chamber experiment with one exception. Fertilizer treatments began one week earlier. This was done to reduce seedling mortality observed during the first growth chamber experiment, which it did. Seedling mortality was decreased from 11.2% to below 2.0% between the two growth chamber experiments (data not shown). The increased dry biomass observed in line 6W-14b#9 however, could not be replicated. This line had similar dry shoot and root weights in both the high and low N treatments when compared to the control plants (Figure 3.8 and 3.9). Lines 6W-4a#5 and 6W-8c#11 also had similar dry shoot and root biomass in the high and low N treatments with one exception. Mean dry root weight of plants form both lines were observed to be significantly smaller than that of the control line when treated with the low N fertilizer.

3.6. Aspartate Aminotransferase Enzyme Activity

The expression of the cytosolic AAT contained within the borders of the T-DNA was driven by the promoter region of the *btg-26* gene of *Brassica napus* (Stroeher et al., 1995). Stroeher et al. (1995) originally showed that *btg-26* can be induced by salt stress.

It was latter shown that the 300 bp promoter region used to express the transgene is tissue specific, directing the expression to root hair epidermis (Good et al., in prep.). To determine if the cytosolic AAT transgene increased the activity of AAT in the root epidermis of salt stressed transgenic plants, a hydroponic experiment was set up. Root and shoot tissues were harvested from transgenic and negative sibling plants either exposed or not exposed to salt stress (inducing or non-inducing conditions respectively) three weeks post germination. Aspartate aminotransferase activity in the roots and the shoots of transgenic plants was compared to the AAT activity in the roots and shoots from each transgenic line's respective negative sibling. Statistical significance could not be determined because the samples were not independently replicated.

A visual inspection of the data and consideration of the variances presented revealed no apparent differences in root AAT activity between any of the transgenic lines and their respective negative sibling when grown under inducing conditions (Table 3.4). These lines included 6W-14b#9, which exhibited an increase in biomass in the first growth chamber experiment, 6W-6a#3, 6W-8c#11, 6W-9a#4, 6W-11b#2, 6W-12d#19, and 6W-13c#18. Furthermore, comparisons between these transgenic lines and their negative siblings failed to yield any noticeable difference for roots grown in non-inducing conditions and shoots grown with or without inducing conditions. Aspartate aminotransferase activity was not determined in line 6W-4a#5 because a corresponding negative sibling line did not exist (see section 3.2.) nor was it determined in line 6W-10a#19 due to time constraints.

3.7. Expression of the Aspartate Aminotransferase Transgene

Since none of the genotypes displayed a clear increase in root AAT activity, the expression of the AAT transgene in the transgenic lines was determined from three week old hydroponically grown plants. Isolated RNA from the roots and shoots was used to create a first strand of transgene specific cDNA that was then amplified using PCR with primers MSASPAT 3' and MSASPAT 5'.

Transcripts from the transgene were detected in roots from line 6W-14b#9 which, was observed to have a biomass increase in the first growth chamber experiment along with lines 6W-4a#5 and 6W-8c#11(Figure 3.10). Expression was also confirmed in shoots of lines 6W-8c#11, and 6W-14b#9 however, expression from the AAT transgene was not as evident. Transgenic AAT transcripts could not be confirmed in shoots of plants from line 6W-4a#5.

In the remainder of the lines analyzed, expression was not as evident (Figure 3.10). These lines included both 6W-9a#4 and 6W-12d#19, which exhibited a decreased and unchanged biomass respectively, in the first growth chamber experiment. The analysis also included lines 6W-11b#2 and 6W-13c#18 that were not screened for a growth phenotype. Transcripts from the AAT transgene could only be detected in the roots of plants from 6W-9a#4.

The initial RT-PCR reactions were carried out at 30 cycles. In order to obtain an estimate of relative abundance of the transcript, the RT-PCR reactions were repeated over a range of PCR cycles. At 20 cycles transgenic AAT transcripts could not be detected in the roots or shoots of any line tested. Increasing the number of cycles above 30 caused significant amounts of contamination from the amplification of genomic DNA templates

despite prior treatment of the RNA samples with DNase. Contamination resulting from genomic DNA could not be separated in the RT-PCR experiment by designing primers to span an intron because the AAT transgene was a cDNA copy of the *Medicago sativa AAT-1* gene. Furthermore, the high degree of DNA sequence homology between known cytosolic AAT genes (Schultz and Coruzzi, 1995; Wadsworth, 1997) limited the ability to design primers that would amplify gene specific products of different sizes. It was reasoned however, that expression of the transgenic AAT gene was low in shoots of all lines tested and in roots of lines 6W-8c#11 and 6W-9a#4 because transcripts could not be amplified above an already low level without amplifying presumed minute amounts of genomic DNA.

Figure 3.1: Results of screening 20 randomly selected T_1 plants from line 6W-14b by amplifying a portion of the selectable marker using PCR, with primers P18 and P19. PCR products were separated using gel electrophoresis and visualized by staining the gel with ethidium bromide. Numbers 1 through 20 represent individual T_1 plants, "+" indicates the positive control (binary vector) and "-" indicates the negative control (aliquot of water).

Table 3.1: Number of insertion events per transgenic line. The ratio of plants containing the transgene (+) to those not (-) was used to identify single insertion events using the chi-squared statistic. Bold type denotes lines advanced to the T_2 generation.

Line ID#	Number of T_1 plants screened	Ratio (+ : -)	Number of Insertion Events
6W-2a	20	19:1	Multiple
6W-2b	20	20:0	Multiple
6W-2c	20	20:0	Multiple
6W-3a	20	20:0	Multiple
6W-5	20	19:1	Multiple
6W-6a	20	15:5	Single
6W-6b	20	18:2	Multiple
6W-6c	20	16:4	Single
6W-7a	20	17:3	Single
6W-7b	20	18:2	Multiple
6W-8a	20	15:5	Single
6W-8c	20	14:6	Single
6W-9a	19	13:6	Single
6W-10a	20	14:6	Single
6W-10b	20	14:6	Single
6W-11a	20	17:3	Single
6W-11b	20	16:4	Single
6W-11c	20	18:2	Multiple
6W-12a	20	17:3	Single
6W-12b	20	16:4	Single
6W-12c	20	19:1	Multiple
6W-12d	20	14:6	Single
6W-13a	20	18:2	Multiple
6W-13b	20	16:4	Single
6W-13c	20	15:5	Single
6W-14b	20	13:7	Single
6W-14c	20	17:3	Single
6W-16a	20	18:2	Multiple
6W-16b	20	20:0	Multiple

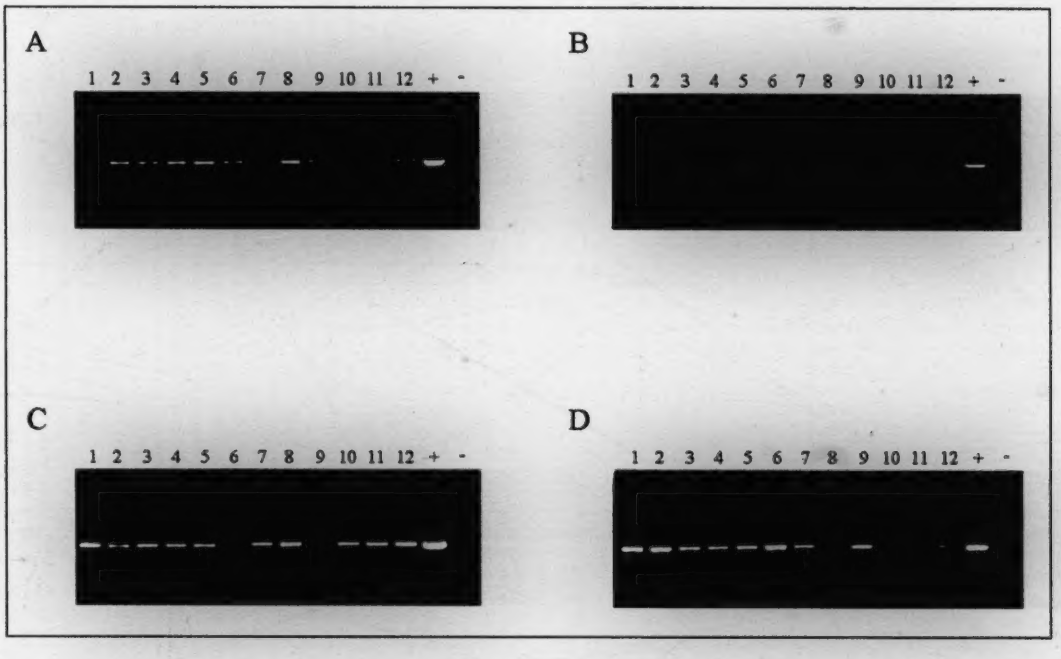

Figure 3.2: Results from screening 12 randomly selected T_2 plants from lines 6W-14b#1 (A), 6W-14b#9 (B), 6W-14b#11 (C), and 6W-14b#13 (D). The presence or absence of the transgene was determined by amplifying a portion of the AAT cDNA contained within the transgene using primers MSASPAT 5' and MSASPAT 3'. Images are ethidium bromide stained gels after electrophoresis. Numbers 1 through 12 represent individual T_1 pants from their respective line, "+" indicates the positive control (binary vector) and "-" indicates the negative control (aliquot of water).

Table 3.2: Identification of T_2 individuals homozygous for the transgene. The ratio of T_2 plants containing an amplifiable portion of the transgene (+) to those not containing the same (-) was recorded for a number of lines. T_2 plants, descended from homozygous T_1 plants, were advanced to the T_3 generation. Advanced lines are indicated by bold type. An "*" denotes lines not screened for a single insertion event.

Line ID#		Ratio (+ : -)	Transgene State
6W-4a# *	**5**	12:0	Homozygous
	7	11:1	Hemizygous
6W-6a#	**3**	12:0	Homozygous
	10	11:1	Hemizygous
6W-7a#	9	11:1	Hemizygous
	11	12:0	Homozygous
6W-8a#	2	12:0	Homozygous
	4	12:0	Homozygous
6W-8c#	3	10:2	Hemizygous
	7	8:4	Hemizygous
	11	12:0	Homozygous
	15	10:2	Hemizygous
6W-9a#	2	6:6	Hemizygous
	3	12:0	Homozygous
	4	12:0	Homozygous

Table 3.2 (continued): Identification of T_2 individuals homozygous for the transgene. The ratio of T_2 plants containing an amplifiable portion of the transgene (+) to those not containing the same (-) was recorded for a number of lines. T_2 plants, descended from homozygous T_1 plants, were advanced to the T_3 generation. Advanced lines are indicated bold type. An "*" denotes lines not screened for a single insertion event.

Line ID#		Ratio (+ : -)	Transgene State
6W-10a#	3	12:0	Homozygous
	5	7:5	Hemizygous
	10	11:1	Hemizygous
	11	12:0	Homozygous
	18	11:1	Hemizygous
	19	12:0	Homozygous
6W-11b#	**2**	12:0	Homozygous
	18	10:2	Hemizygous
6W-12d#	2	8:4	Hemizygous
	19	12:0	Homozygous
6W-13b#	**4**	12:0	Homozygous
	5	11:1	Hemizygous
	13	11:1	Hemizygous
6W-13c#	1	11:1	Hemizygous
	18	12:0	Homozygous
6W-14a# *	**1**	12:0	Homozygous
6W-14b#	1	11:1	Hemizygous
	9	12:0	Homozygous
	11	10:2	Hemizygous
	13	9:3	Hemizygous

Table 3.3: Transgenic lines and their corresponding negative siblings. Select lines were confirmed as transgenic or non-transgenic by scoring the T_3 progeny for resistance to kanamycin. Fifteen to 20 seeds were germinated on half strength Murashige and Skoog basal medium containing 150μg/ml kanamycin. After two weeks the percentage of kanamycin resistant (Kan^R) seedlings was recorded for each line. No kanamycin resistant seedlings were observed for N-o-1. (data not shown). "*" indicates that a corresponding negative sibling does not exists because a T_1 plant not containing the transgene did not segregate out in the T_1 progeny. "**" denotes negative siblings lines that were ended because they failed to produce viable seed.

Transgenic Lines	Kan^R Seedlings (%)	Negative Siblings	Kan^R Seedlings (%)
6W-4a#5	90 (9/10)	*	*
6W-6a#3	nd	6W-6a#4	nd
6W-7a#11	nd	**	**
6W-8a#4	nd	6W-8a#16	nd
6W-8c#11	100 (15/15)	6W-8c#10	25 (4/16)
6W-9a#4	70 (7/10)	6W-9a#12	0 (0/13)
6W-10a#19	nd	6W-10a#20	nd
6W-11b#2	94 (15/16)	6W-11b#11	nd
6W-12d#19	82 (9/11)	6W-12d#20	0 (0/17)
6W-13b#4	nd	6W-13b#16	nd
6W-13c#18	100 (18/18)	6W-13c#12	0 (0/17)
6W-14a#1	100 (18/18)	*	*
6W-14b#9	nd	6W-14b#15	16 (3/19)

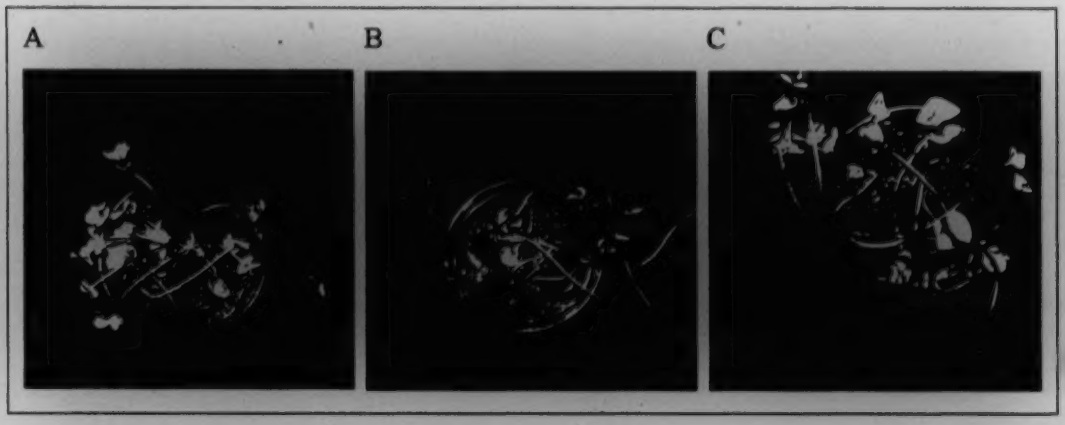

Figure 3.3: Bioassay for NPT II expression in 6W-12d#19. T_3 seeds from *Brassica napus*, N-o-1 (A), 6W-12d#19 (B), and 6W-12d#20 (negative sibling of 6W-12d#19) (C) were germinated on half strength Murashige and Skoog basal medium containing 150µg/ml kanamycin for two weeks.

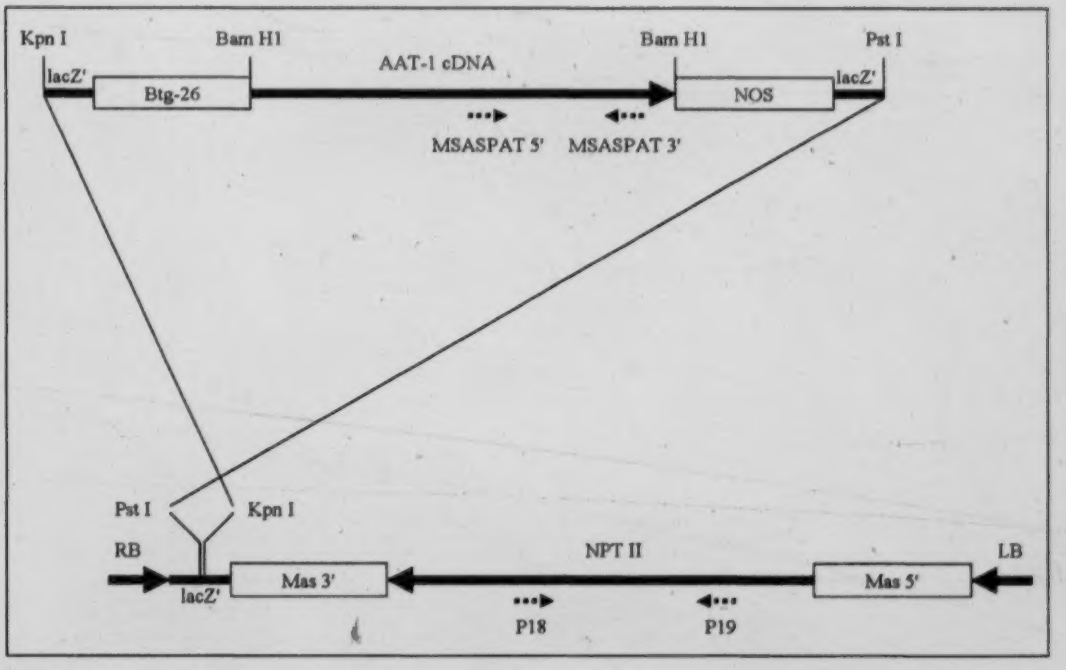

Figure 3.4: Organization of genes located between the left and right borders of the binary vector p26gAspATNS47. The locations of primer binding sites used to screen the transgenic lines are indicated by dashed arrows. Primer pairs MSASPAT 5' and MSASPAT 3' and P18 and P19 amplify 489 and 657 bp fragments respectively.

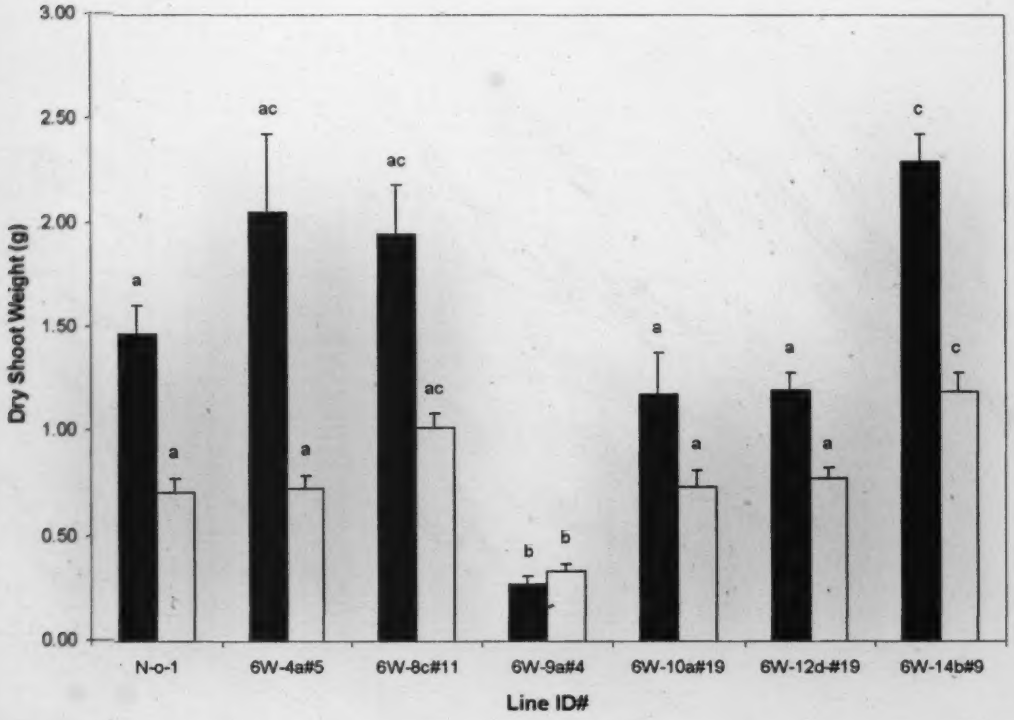

Figure 3.5: Dry shoot biomass of independent transgenic lines fertilized with a high nitrogen fertilizer (black bars) or a low nitrogen fertilizer (white bars) in experiment one. Fertilizer treatments began two weeks after planting and shoots were harvested four weeks after fertilizer treatments began. Values are means of three to five T_2 plants. Significantly different means in the high ($F_{6,23}=14.01$, $p<0.0005$) and low ($F_{6,20}=17.15$, $p<0.005$) nitrogen fertilizer treatments respectively were determined using Tukey's multiple comparisons test and are indicated with the lower case letters a, b, and, c. Error bars are standard error. N-o-1 was the control all lines were compared to.

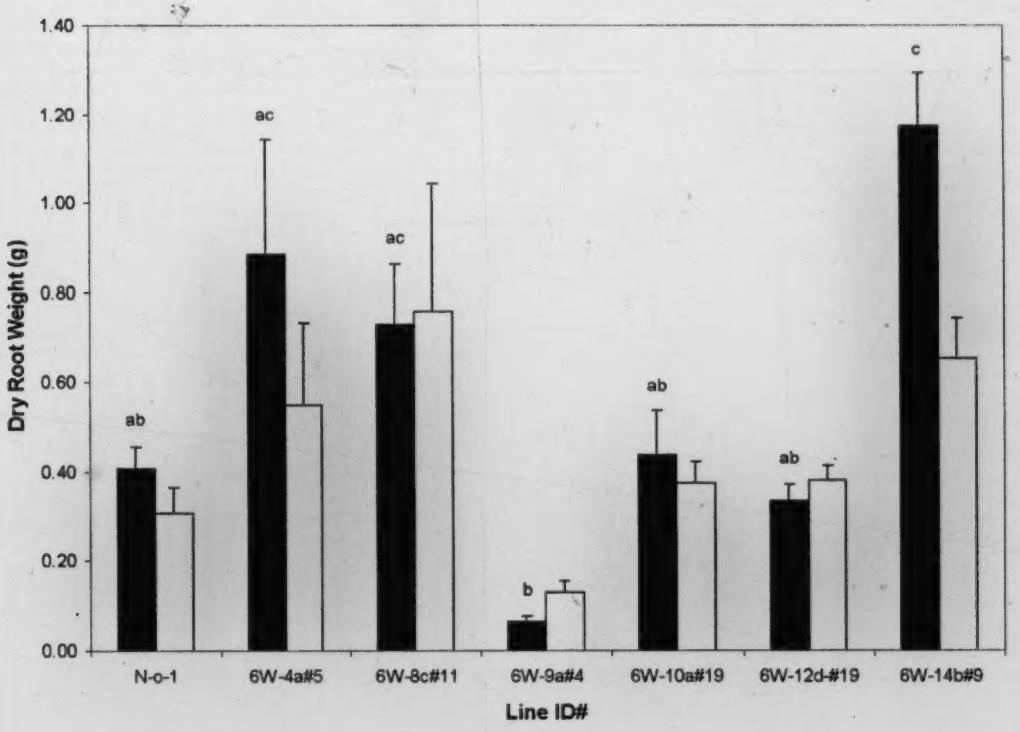

Figure 3.6: Dry root biomass of independent transgenic lines fertilized with a high nitrogen fertilizer (black bars) or a low nitrogen fertilizer (white bars) in experiment one. Fertilizer treatments began two weeks after planting and roots were harvested four weeks after fertilizer treatments began. Values are means of three to five T_2 plants. Significantly different means in the high nitrogen fertilizer treatment ($F_{6,23}$=11.65, $p<0.0005$) were determined using Tukey's multiple comparisons test and are indicated with lower case letters a, b, and, c. No statistically significant differences exist between the dry root biomass of lines treated with the low nitrogen fertilizer ($F_{6,20}$=2.26, p=0.079). Error bars are standard error. N-o-1 was the control all lines were compared to.

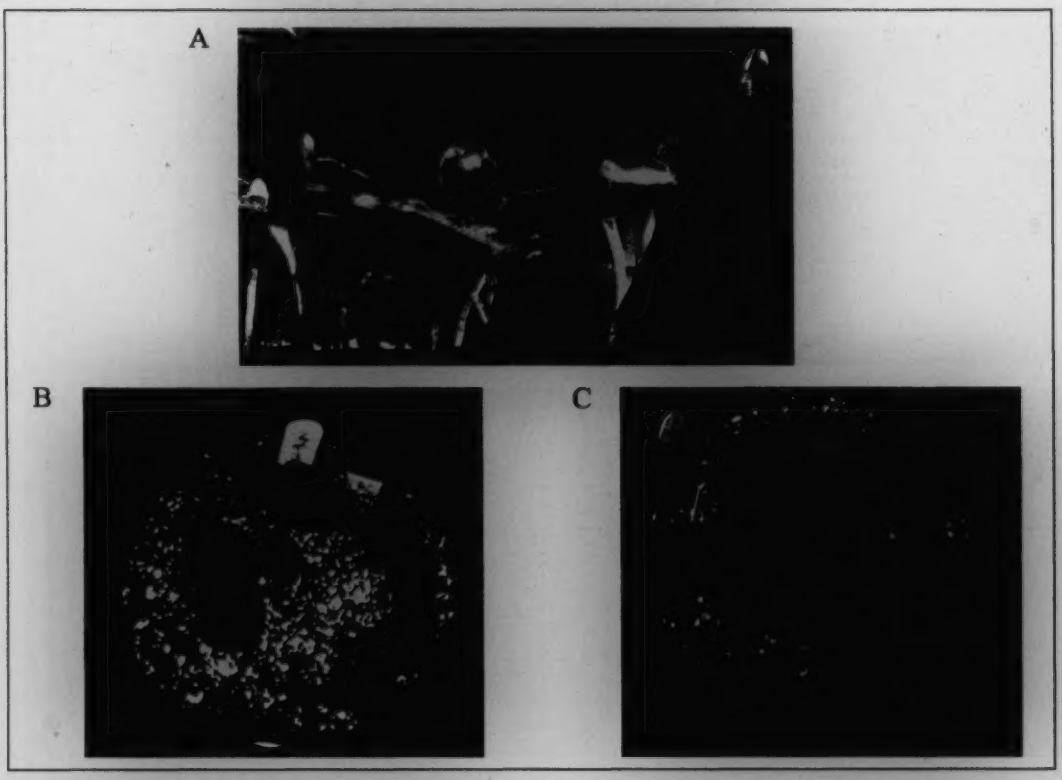

Figure 3.7: Aberrant phenotype of T_3 individuals from line 6W-9a#4. Panel A shows the mutant leaf morphology in true leaves (red arrows). Panel B shows the stunted phenotype of a T_3 individual from line 6W-9a#4 compared to an individual from a corresponding negative sibling (panel C). Both plants were germinated at the same time in 15 cm diameter pots.

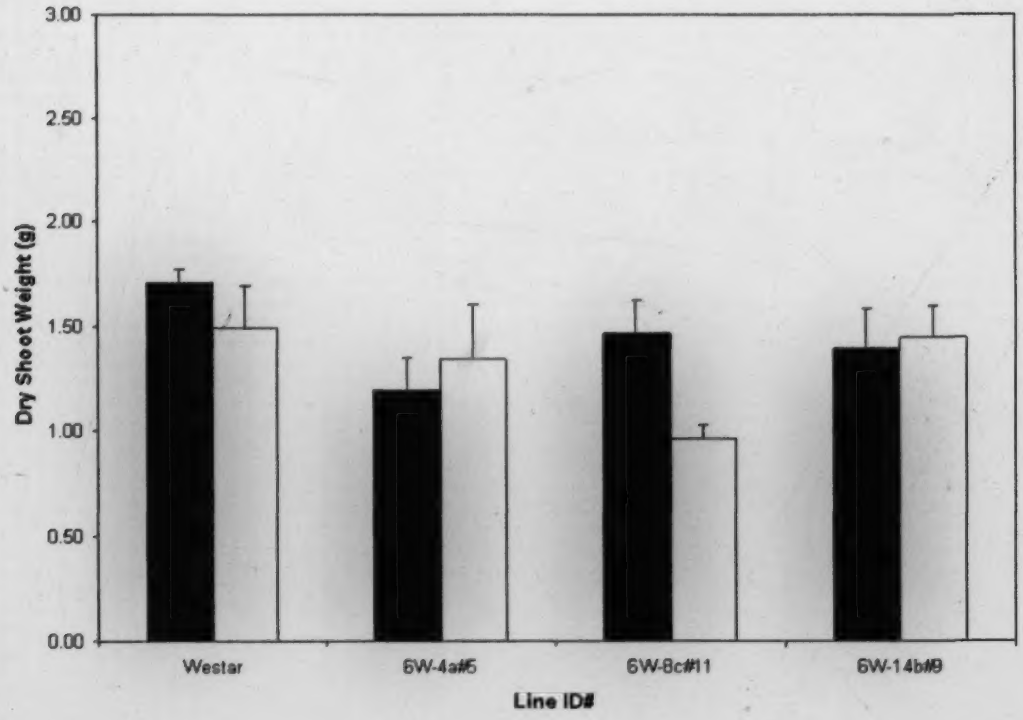

Figure 3.8: Dry shoot biomass of lines treated with high N fertilizer (black bars) and low N fertilizer (white bars) in experiment two. Fertilizer treatments began one week after planting and shoots were harvested four weeks after fertilizer treatments began. Values are means of three to five T_2 plants. No statistically significant differences exist between the dry shoot biomass of lines treated with the high nitrogen fertilizer ($F_{3,15}$=1.73, p=0.204) and the low nitrogen fertilizer respectively ($F_{3,15}$=2.11, p=0.142). Error bars are standard error. N-o-1 was the control all lines were compared to.

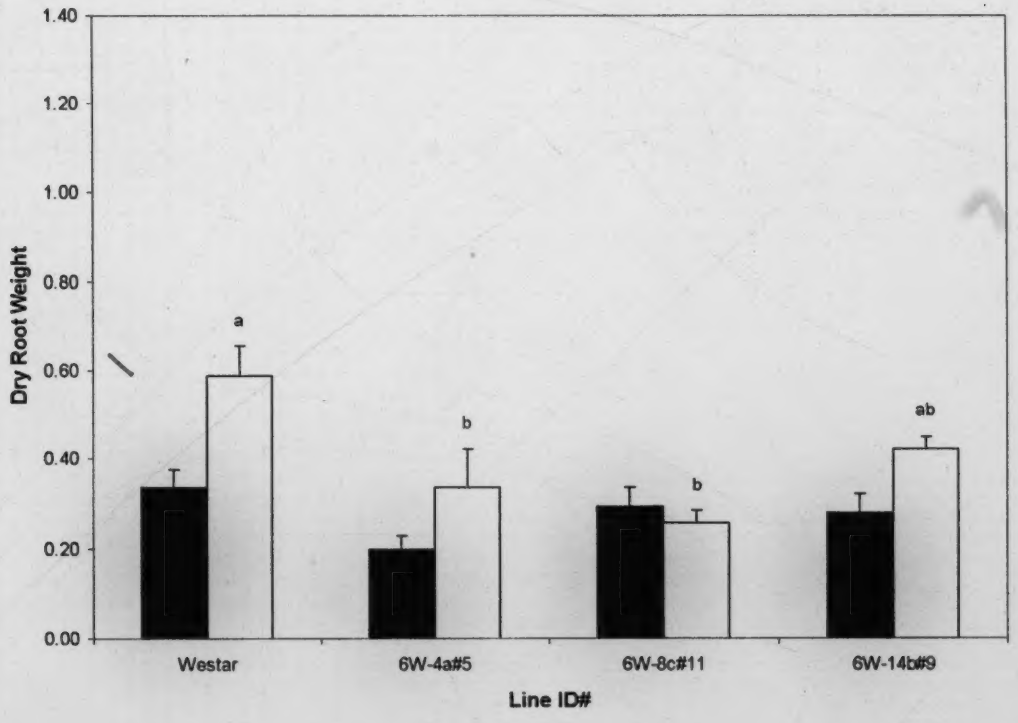

Figure 3.9: Dry root biomass of lines treated with high N fertilizer (black bars) and low N fertilizer (white bars) in experiment two. Fertilizer treatments began one week after planting and shoots were harvested four weeks after fertilizer treatments began. Values are means of three to five T_2 plants. No statistically significant differences exist between the dry root biomass of lines treated with the high nitrogen fertilizer ($F_{3,15}$=2.28, p=0.124) but did exist for lines treated with the low nitrogen fertilizer ($F_{3,15}$=8.43, p=0.002). Significantly different means in the low nitrogen fertilizer treatment were determined using Tukey's multiple comparisons test and are indicated with lower case letters a and b. Error bars are standard error. N-o-1 was the control all lines were compared to.

Table 3.4: Aspartate aminotransferase enzyme activity in induced and non-induced roots and shoots of hydroponically grown T_3 plants. Mean activity from three biological replicates plus or minus the standard error is shown. The addition of 100mM NaCl to the liquid medium was used to induce the transgene. A statistical analysis could not be completed because the samples were not independently replicated.

Line		Activity (μmoleNADH/min*mg soluble protein X 1000)			
		Roots		Shoots	
		Non-inducing	Inducing	Non-inducing	Inducing
6W-6a	Transgenic (#3)	0.80 ± 0.04	1.11 ± 0.04	0.27 ± 0.01	0.28 ± 0.01
	Negative Sibling (#4)	0.83 ± 0.01	1.17 ± 0.04	0.26 ± 0.01	0.27 ± 0.01
6W-8c	Transgenic (#11)	0.77 ± 0.06	0.54 ± 0.27	0.22 ± 0.01	0.33 ± 0.03
	Negative Sibling (#10)	0.86 ± 0.04	0.19 ± 0.03	0.23 ± 0.01	0.45 ± 0.03
6W-9a	Transgenic (#4)	0.87 ± 0.02	0.91 ± 0.08	0.51 ± 0.01	0.24 ± 0.02
	Negative Sibling (#12)	1.04 ± 0.05	1.03 ± 0.04	0.44 ± 0.03	0.27 ± 0.02
6W-11b	Transgenic (#2)	0.71 ± 0.03	1.05 ± 0.11	0.23 ± 0.01	0.23 ± 0.01
	Negative Sibling (#11)	0.79 ± 0.01	1.08 ± 0.03	0.23 ± 0.01	0.23 ± 0.01
6W-12d	Transgenic (#19)	0.79 ± 0.07	0.76 ± 0.01	0.34 ± 0.01	0.23 ± 0.01
	Negative Sibling (#20)	0.78 ± 0.08	0.78 ± 0.02	0.33 ± 0.02	0.22 ± 0.01
6W-13c	Transgenic (#18)	0.96 ± 0.06	1.00 ± 0.03	0.30 ± 0.02	0.28 ± 0.02
	Negative Sibling (#12)	0.95 ± 0.08	0.94 ± 0.04	0.32 ± 0.02	0.33 ± 0.07
6W-14b	Transgenic (#9)	0.76 ± 0.03	0.87 ± 0.25	0.23 ± 0.01	0.27 ± 0.13
	Negative Sibling (#15)	0.77 ± 0.01	0.95 ± 0.12	0.22 ± 0.01	0.33 ± 0.03

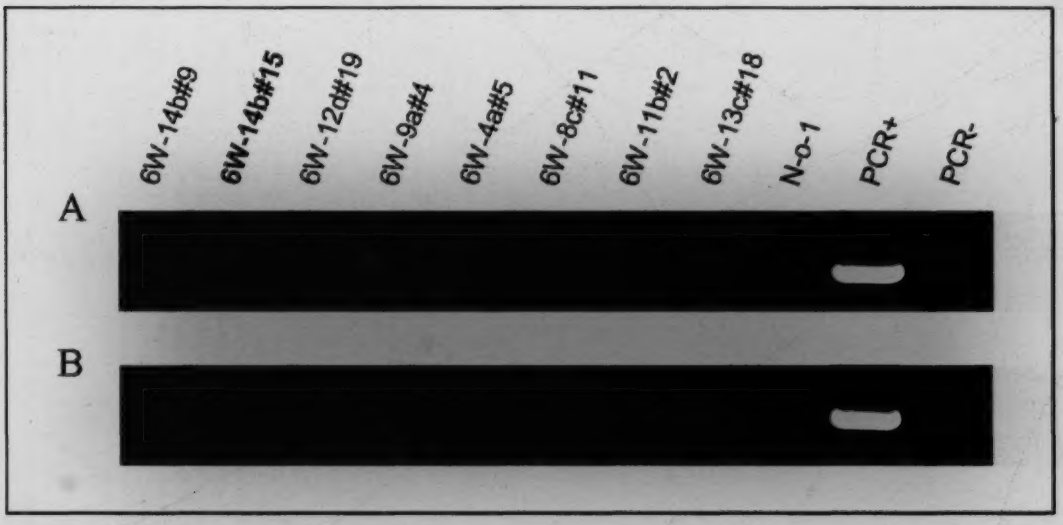

Figure 3.10: Presence of transgenic AAT transcripts in shoots (A) and roots (B) of independent transgenic lines. Thirty cycles of RT-PCR were used to amplify AAT transgene transcripts from 2.5µl of total RNA extracted from induced, hydroponically grown plants. Primers specific for the AAT transgene were used to synthesis the first strand cDNA and to amplify the cDNA template. 6W-14b#15, indicated in bold, is the negative sibling of 6W-14b#9. N-o-1 is the double haploid line derived from *B. napus* cv Westar. The template for the PCR+ reaction was the binary vector. No PCR template was included in the PCR- sample.

4. Discussion

4.1. Screening Putative Transgenic Plants

Agrobacterium mediated transformation of *Brassica napus*, N-o-1 with the binary vector p26gAspATNS47 resulted in 37 T_0 transgenic plants which were Kan^R and contained an amplifiable portion of the selectable marker gene (NPT II). Of these 37 lines, 17 were identified containing either a single T-DNA or multiple T-DNA copies inserted at a single locus within the genome (single insertion event) using a PCR screen of the T_1 generation (Table 3.1). Eleven of the 17 T_0 lines with single insertion events were advanced to the next generation on the basis of seed vigor, ensuring that the greatest number of independent insertion events were available for analysis.

An additional five lines were also advanced to the T_2 generation. The number of insertions in each of these lines could not be determined due to a limited number of viable T_1 seeds. These lines could contain a single transgene at a single locus, multiple transgenes at a single locus, a single transgene at multiple loci, or multiple transgenes at multiple loci. These lines were advanced because of the possibility that they represented novel insertion events.

Transgenic plants with a single transgene are desirable because the possibility of copy number (Bhat and Srinivasan, 2002; Tang et al., 2003) or gene silencing (Matzke et al., 1989; Vaucheret and Fagard, 2001) influencing transgene expression is greatly reduced. Unfortunately, the PCR screen used cannot distinguish between a single transgene inserting at a single locus or multiple transgenes inserting at a single locus. A Southern blot would be required to differentiate between the two scenarios. DNA

extracted from a single line would first need to be digested with a restriction endonuclease that cuts once within the transgene. The number of transgene copies integrated into the genome can then be determined by probing the membrane with a probe that spans the restriction site. Two novel bands indicate a single transgene whereas three or more novel bands indicate multiple transgenes. Furthermore, variation in the size of the bands between lines could have been used to demonstrate that the 16 lines represent independent insertion events. This analysis was not done due to time constraints but it is strongly recommended that the Southern analysis be completed in order to characterize each insertion event. Nonetheless, the PCR screen reduced the number of lines to be characterized to a manageable number while identifying those lines with the highest probability of containing a single transgene.

Homozygous lines were selected from the segregating T_2 generation again using a PCR based approach, this time by amplifying a portion of the AAT transgene from each of the 16 T_1 lines advanced to the T_2 generation. Homozygous lines eliminate the time required to identify transgenic from non-transgenic plants compared to lines still segregating for the transgene and eliminate dosage effects of the transgene between lines homozygous for the transgene and lines hemizygous for the transgene (Beaujean et al., 1998; Azhakanandam et al., 2000). A T_1 plant homozygous for the transgene was identified for 13 of the 16 transgenic lines screened (Table 3.2). All 13 lines were advanced to the T_3 generation.

It should be noted that a small (3.2%) chance exists that a line hemizygous for the transgene was falsely identified as being homozygous for the transgene with the screening protocol employed. This probability can be decreased in future protocols by

increasing the sample size used to screen the T_2 plants. However, a sample size of 12 represents a reasonable number of plants to screen per line with an acceptably low probability of classifying a line hemizygous for the transgene when in fact it is homozygous.

Overall, the PCR based screening procedures identified lines 6W-4a#5, 6W-6a#3, 6W-7a#11, 6W-8a#4, 6W-8c#11, 6W-9a#4, 6W-10a#19, 6W-11b#2, 6W-12d#19, 6W-13b#4, 6W-13c#18, 6W-14a#1, and 6W-14b#9 as being homozygous for the transgene and, with the exception of 6W-4a#5 and 6W-14a#1, containing a single insertion event within their respective genomes (Table 3.2). Corresponding negative siblings were identified for each line from the segregating T_1 generation with notable exceptions including lines 6W-4a#5, 6W-7a#11, and 6W-14a#1 (Table 3.3). The negative siblings where chosen as the control plant because they were easy to identify during the screening procedure and they can control for potential variation caused by both the initial germplasm and somaclonal variation induced by the tissue culture process (Jain, 2001; Bhat and Srinivasan, 2002). All of the above transgenic lines and their respective negative siblings were advanced to the T_3 generation to increase seed for further characterization.

4.2. Neomycin Phosphotransferase II Bioassay

Results from the neomycin phosphotransferase II (NPT II) assay suggest that either the screening procedures used to identify homozygous lines, the screening procedures used to identify negative siblings, or both were unreliable (Table 3.3). Of the seven transgenic lines screened for NPT II activity, only lines 6W-8c#11, 6W-13c#18,

and 6W-14b#9 were confirmed to be homozygous. The remaining four lines appeared to be hemizygous for the transgene based on the appearance of both kanamycin resistant (Kan^R) and kanamycin sensitive (Kan^S) T_3 seedlings. Additionally, two out of the five negative siblings identified in the original screen contained Kan^R T_3 seedlings. The progeny from a true negative sibling would all be Kan^S.

However, it seems unlikely that four out of seven lines analyzed were falsely identified as homozygous lines considering the low probability (3.2%) of mistaken identification occurring using the original screening conditions. I speculate that a low level of expression from the selectable marker in lines 6W-4a#5, 6W-9a#4, 6W-11b#2, and 6W-12d#16 (not determined) coupled with the moderate concentration of kanamycin used, could explain the bioassay results. Low NPT II expression would allow plants containing the selectable marker to appear as Kan^S at moderate to high kanamycin concentrations while low to moderate kanamycin concentrations may allow non-transgenic plants to appear as Kan^R. Variation in NPT II expression could occur via a position effect of the transgene (Peach and Velten, 1991) and the observation of Kan^R seedlings in the negative sibling lines could imply that the kanamycin concentration used for the bioassay was not stringent enough. Either of these explanations, individually or in combination, may explain the discrepancy between the screening data and the bioassay results.

The phenomenon that transgenic lines containing a few T_3 Kan^S seedlings had corresponding negative sibling lines with no Kan^R T_3 seedlings whereas transgenic lines containing no T_3 Kan^S seedlings had corresponding negative siblings with a few Kan^R T_3 seedlings still can not be accounted for (Table 3.3). Position effect variegation, the

concentration of kanamycin used in the NPT II assay, or both may contribute to the phenomenon. On the other hand, the result could be an artifact of the experiment. The significance of this observation is unknown.

Other groups have recently screened *Brassica* seedlings by germinating seeds on medium containing kanamycin (Ponstein et al., 2002; Cardoza and Stewart, 2003). The results presented by Cardoza and Stewart (2003) indicated that they did not encounter escapes, however they did not report the concentration of kanamycin, the medium, or the growth conditions used to screen the plants. On the other hand, Ponstein et al. (2002) found that distinguishing between Kan^R and Kan^S *B. napus* plants by germinating seeds on non-nutritive medium containing 50 μg/ml of kanamycin was not reliable even after the plants had been on the medium for two to three weeks. The results of the NPT II bioassay in this thesis agree with Ponstein et al. (2002) findings. It is therefore not recommended to rely solely on this type of germination experiment when attempting to screen *B. napus* plants for the presence of an NPT II gene. A PCR based approach would be more reliable especially if a large sample (greater than 20) was screened.

4.3. Growth Chamber Experiments

Transgenic line 6W-14b#9 showed an increased root and shoot biomass relative to control plants in the first growth chamber experiment (Figure 3.5 and 3.6). The over-expression of the AAT transgene may have contributed to the observed biomass increase because of the role cytosolic AAT plays in primary plant nitrogen (N) metabolism. Aspartate aminotransferase is known to catalyze the transamination reaction involving the production of aspartate (Asp) and 2-oxoglutarate from glutamate and oxaloacetate.

The aspartate created by cytosolic forms of AAT is used as an N transport molecule during the day and when carbon (C) skeletons are abundant (Shultz et al., 1998; Miesak and Coruzzi, 2002). Aspartate can also be converted to asparagine (Asn) by asparagine synthetase (AS) in order to continue the flow of N throughout the plant. Asparagine represents a more efficient N transport molecule than Asp by virtue of its high N to C ratio. However, AS only catalyzes the conversion of Asp to Asn in the dark or when C skeletons become limiting, presumably due to the energy required to convert Asp to Asn by AS (Lam et al., 1998; Miesak and Coruzzi, 2002; Wong et al., 2004). The observed increase in dry biomass could have resulted from an increase in AAT activity which caused an increase in primary N assimilation. The increase in primary N assimilation may have been triggered by an increase in AAT activity which resulted in increased pools of N transport molecules, specifically Asp and Asn. The fact that the AAT enzyme assays failed to distinguish AAT activity differences between the transgenic lines and their respective negative sibling due to the sensitivity of the assay (see section 4.5.), leaves this theory as only speculation.

Furthermore, an attempt to replicate the increased biomass observed in line 6W-14b#9 was unsuccessful (Figure 3.8 and 3.9). It is noted that the fertilizer treatments began one week earlier in the second growth chamber experiment but it would be highly unlikely that this change alone could account for the discrepancy in plant biomass between the two experiments. Replicating the results is imperative prior to making further predictions as to the cause of a biomass increase in the transgenic lines, if an increase actually exists. It is therefore recommended that a third growth chamber

experiment be carried out to demonstrate whether the biomass increase observed in line 6W-14b#9 is real.

The remaining lines tested for increased growth in experiment one were 6W-4a#5, 6W-8c#11, 6W-9a#4, 6W-10a#19, and 6W-12d#19. Plants from line 6W-9a#4 exhibited a decrease in dry shoot and root weight (Figure 3.5 and 3.6) that is speculated to result from either an insertional mutation caused by the transgene or somaclonal variation rather than the over-expression of the transgene. Individuals were stunted, had poor seed set, and an aberrant leaf morphology compared to both *B. napus*, N-o-1 and its negative sibling (Figure 3.7). On the other hand, no observed biomass differences existed between lines 6W-4a#5, 6W-8c#11, 6W-10a#19, and 6W-12d#19 when compared to the control (Figure 3.5 and 3.6). The transgene in these lines was either not over-expressed or over-expression had no noticeable effect on biomass. Both of these scenarios have been previously reported in prior attempts to alter specific plant characteristics by the over-expression of a transgene (Levesque-Lemay et al., 2003; Lucker et al., 2004; Mazel et al., 2004). However, it is recognized that a variety of factors could have affected the phenotype of individuals from lines 6W-4a#5, 6W-8c#11, 6W-9a#4, 6W-10a#19, and 6W-12d#19. The goal of the thesis though, was to identify nitrogen efficient lines and as such the specific cause of the decreased biomass in line 6W-9a#4 or the unchanged biomass in lines 6W-4a#5, 6W-8c#11, 6W-10a#19, and 6W-12d#19 were not investigated further.

Finally, seedling mortality observed in the first growth chamber experiment was believed to be caused by the extended period that nutrients were withheld from the plants. Seed reserves are required for seedling metabolism before the seedlings become

photosynthetically active (Bewley and Black, 1994). Once reserves are depleted and seedlings become photosynthetically active, a lack of macronutrients and/or micronutrients will cause a nutrient deficiency, which if not addressed will result in plant death. The decrease in seedling mortality observed in the second growth chamber experiment implies that seedlings did not experience the degree of nutrient deficiency experienced in the first growth chamber experiment. In the second growth chamber experiment, the seed reserves in the control line and transgenic lines were large enough to support seedling growth and development until the fertilizer treatment began.

4.4. Putative NUEg Transgenic Lines

A central goal of this thesis was to identify transgenic lines of *B. napus* that were nitrogen use efficient in yield (NUEg). Moll et al. (1982) defined NUEg as plant yield per unit N applied. Since seed production or yield was not measured in these plants nor can it be accurately measured in a growth chamber experiment, I was unable to determine NUEg. However, biomass can be used as an indicator of yield because it is generally recognized that increases to biomass result in corresponding yield increases. Thus, for the purpose of identifying potential candidate NUEg genotype in this thesis I define the term biomass nitrogen use efficiency (NUEb) as a plant's biomass per unit N applied. Based on this definition, line 6W-14b#9, which showed an increase in root and shoot biomass at the two applied N rates, could be NUEb genotypes. This increase however, was not replicable and until such time that the increase is conclusively demonstrated the conclusion that these lines are NUEb must be viewed with caution.

Had any line conclusively shown an increase in plant biomass it would represent a putative NUEg *B. napus* genotype, assuming that increased biomass is positively correlated with yield. A modified growth chamber experiment could confirm this by growing the plants to maturity and determining yield to provide a direct measure of NUEg. Furthermore, increasing the number of N fertilizer rates tested may contribute to understanding how the transgenic lines responded to different N fertilizer rates. Plotting the relationship of yield versus applied N over different nitrogenous fertilizer rates will generate an N growth response curve where NUEg is the slope of the function (Figure 1.4). Curves representing increased NUEg resulting from a decrease in applied N without a proportional decrease in yield represent agronomically desirable NUEg genotypes (Figure 1.6). Only this type of approach, employed in a field trial, can effectively identify NUEg genotypes of significant agronomical value. Time did not allow for an analysis of yield in the transgenic lines nor were field trials conducted.

4.5. Aspartate Aminotransferase Activity Assays

The transgene consisted of a cytosolic cDNA copy of a *Medicago sativa* AAT gene (AAT-1). The expression of the transgene was driven by an inducible (Stroeher et al., 1995) and root hair, epidermal specific promoter region of *btg-26* (Good et al., in prep.). To establish if ectopic over-expression from the transgene increased AAT enzyme activity *in vivo*, AAT enzyme activity in the roots and shoots of transgenic plants and their corresponding negative siblings was determined. No transgenic line appeared to have increased AAT activity in roots relative to their respective negative sibling (Table 3.6). However, the fact that the samples in this experiment were not independently

replicated prohibited the use of a statistical test to confirm this observation. Future experiments in which each sample is independently replicated would be required in order to make unambiguous conclusions about the nature of AAT activity between a transgenic line and its respective negative sibling.

None-the-less, two explanation can be put forth to explain the apparent lack of an observable increase of AAT activity in roots of transgenic plants grown under inducing conditions. The first is that none of the lines have increased AAT activity. The second is that the approach used to detect AAT activity was not sensitive enough to detect an increase. Transcripts from the transgenic AAT accumulate in the epidermis (Good et al., in prep.) but total root homogenates were used in the AAT enzyme assays. Root hairs contribute only a small fraction of the total homogenate. Furthermore, plants in general contain a number of different endogenous isoenzymes of AAT that are active in roots (Lam et al., 1996; Wadsworth, 1997; Liepman and Olsen, 2004). Assuming that the majority of these transcripts are transcribed into functional AAT enzymes that contribute to root AAT activity, then only a very large increase in AAT activity in root hair epidermis would be detected above background levels. It seems likely that if there was an increase in AAT activity in roots hairs, the signal to noise ratio is sufficient to obscure any differences between a transgenic line and its negative sibling.

Determining the AAT enzyme activity in a purified root hair fraction could support the prediction that the AAT transgene increases root epidermal AAT activity. A system designed to promote the growth of root hairs and allow them to be harvested has been previously described (Lauter at al., 1996). Unfortunately, due to time and material limitations, this system could not be reproduced. If this method detected an increase in

AAT activity in roots, particularly in lines exhibiting increased biomass, this would support the hypothesis that over-expression of the cytosolic AAT transgene in *B. napus* can increase AAT activity *in vitro*.

4.6. Transgenic Aspartate Aminotransferase Expression Analysis

Expression of the transgenic AAT-1 cDNA was confirmed in roots of 6W-14b#9 plants observed to have an increase in biomass in the first growth chamber experiment, using an end point RT-PCR approach (Figure 3.10); however the specific level of expression was not quantitatively determined. A strong, positive correlation between biomass of transgenic plants and root hair specific expression of the AAT transgene from the parental plant would support the hypothesis that ectopic over-expression of a cytosolic AAT gene can increase tissue specific AAT activity.

The expression analysis also indicated a low level of AAT transgene expression in shoots relative to roots. This observation supports the tissue specific pattern of gene expression previously shown for the *btg-26* promoter (Good et al., in prep.). It is acknowledged, however, that the attempt to quantify transgenic AAT transcripts is preliminary. In future work, confirmation of the level of ectopic over-expression of the AAT transgene should be demonstrated by quantifying transcript levels in total RNA isolated specifically from root hairs, using either an quantitative RT-PCR approach or Northern blots.

It can be concluded though, that this method of over-expressing a cytosolic AAT gene in a tissue specific manner could increase the cytosolic AAT activity in root hairs assuming that translational and post-translational regulation does not limit AAT activity.

No reports documenting any form of translational or post-translational regulation of plant AAT genes have been reported in the literature.

5. Conclusion

5.1. Goals of this Thesis

The goal of this research was to develop nitrogen use efficient germplasm of *Brassica napus* through genetic engineering. The hypothesis tested was that ectopic over-expression of a *Medicago sativa* cytosolic aspartate aminotransferase (AAT) cDNA in root hairs of *B. napus* would increase grain nitrogen use efficiency (NUEg) in the transgenic lines. The basis for this hypothesis was the demonstration that NUEg was increased by the same tissue-specific over-expression of a barley alanine aminotransferase cDNA in *Brassica napus* (Good et al., in prep.). The specific goals of the thesis included:

- The creation of a number of independent transgenic lines that contain a single insertion event.
- The identification of a series of homozygous transgenic lines from the independent transgenics that contain a single insertion event.
- A preliminary analysis of nitrogen efficiency in the selected transgenic lines.
- The characterization of AAT activity and expression in the selected transgenic lines.

5.2. Independent Transgenic Lines

Of the 37 T_0 transgenic plants created by an *Agrobacterium* mediated transformation procedure, 17 plants were identified as containing a single insertion event

of the T-DNA within their respective genome. Eleven of these 17 T_0 plants were advanced to the T_3 generation in order to identify homozygous individuals for each line and to increase the amount of seed for further analysis. Another two transgenic lines were advanced to the T_3 generation for further analysis because they represented additional independent insertion events. Although they were not screened for the number of insertion events, homozygous individuals from these lines were selected. In total 13 independent T_3 transgenic lines containing a single insertion event and that were homozygous for the transgene were identified and characterized.

5.3. Nitrogen Efficient *Brassica* Genotypes

Line 6W-14b#9 was observed to have an increase in shoot and root biomass in the first growth chamber experiment when treated with two nitrogenous fertilizer amounts. This line may represent a biomass nitrogen use efficient (NUEb) line. Unfortunately, a second growth chamber experiment could not replicate the biomass increases initially observed in 6W-14b#9 in the first growth chamber experiment. Until such time that the biomass increases are replicated, 6W-14b#9 can only be tentatively considered NUEb genotypes.

It would be desirable to not only identify NUEb genotypes but also to identify any NUEg lines that do not display a decrease in yield when there is a decrease in applied nitrogen. These lines are considered to have a high agronomic value and environmental benefits because they would not only have yields equivalent to the best available varieties, but would also allow for the reduction of applied nitrogenous fertilizer. I

believe screening for these lines at a number of nitrogenous fertilizer rates in a field trial represents the best approach to identify these NUEg genotypes.

5.4. Characterization of Putative Nitrogen Efficient Lines

A number of transgenic lines were characterized for AAT activity. Contrary to predictions, no increase was observed in roots of any of the transgenic line grown under inducing conditions. This was believed to be due to the inability of the enzyme assay to detect an increase in AAT activity, resulting from the transgene, above background levels in whole root homogenates. Enzyme activity measurements on isolated root hairs should be used to detect an increase in AAT activity in root hairs of transgenic plants in future experiments.

Transgenic AAT expression was also analyzed in several of the transgenic lines. Expression was confirmed in roots of line 6W-14b#9, which showed an increase in biomass in the first growth chamber experiment, and lines 6W-4a#5, 6W-8c#11, and 6W-9a#4. Shoot expression was not evident; however this observation is in accordance with the observed expression pattern of the *btg-26* promoter used to drive expression of the AAT transgene (Stroeher et al., 1995; Good et al. in prep). Expression of the AAT transgene should still be accurately quantified in both root and shoot tissues using either a semi-quantitative RT-PCR or Northern blot approach.

5.5. Future Work

In the future, there are a number of experiments that need to be completed to more fully characterize these transgenic lines. First, there needs to be an attempt to correlate

the expression levels of the transgene with increased biomass in the transgenics. This would allow me to determine if specific ectopic expression patterns of the AAT transgene could increase nitrogen use efficiency. Second, while increases in biomass may well be correlated with an increase in yield, this needs to be determined experimentally. Recent data in my laboratory indicate that this may not always be the case (Shrawat et al. unpublished). Finally, it would be useful to determine what metabolic changes occur in the transgenic lines which display an NUEb phenotype.

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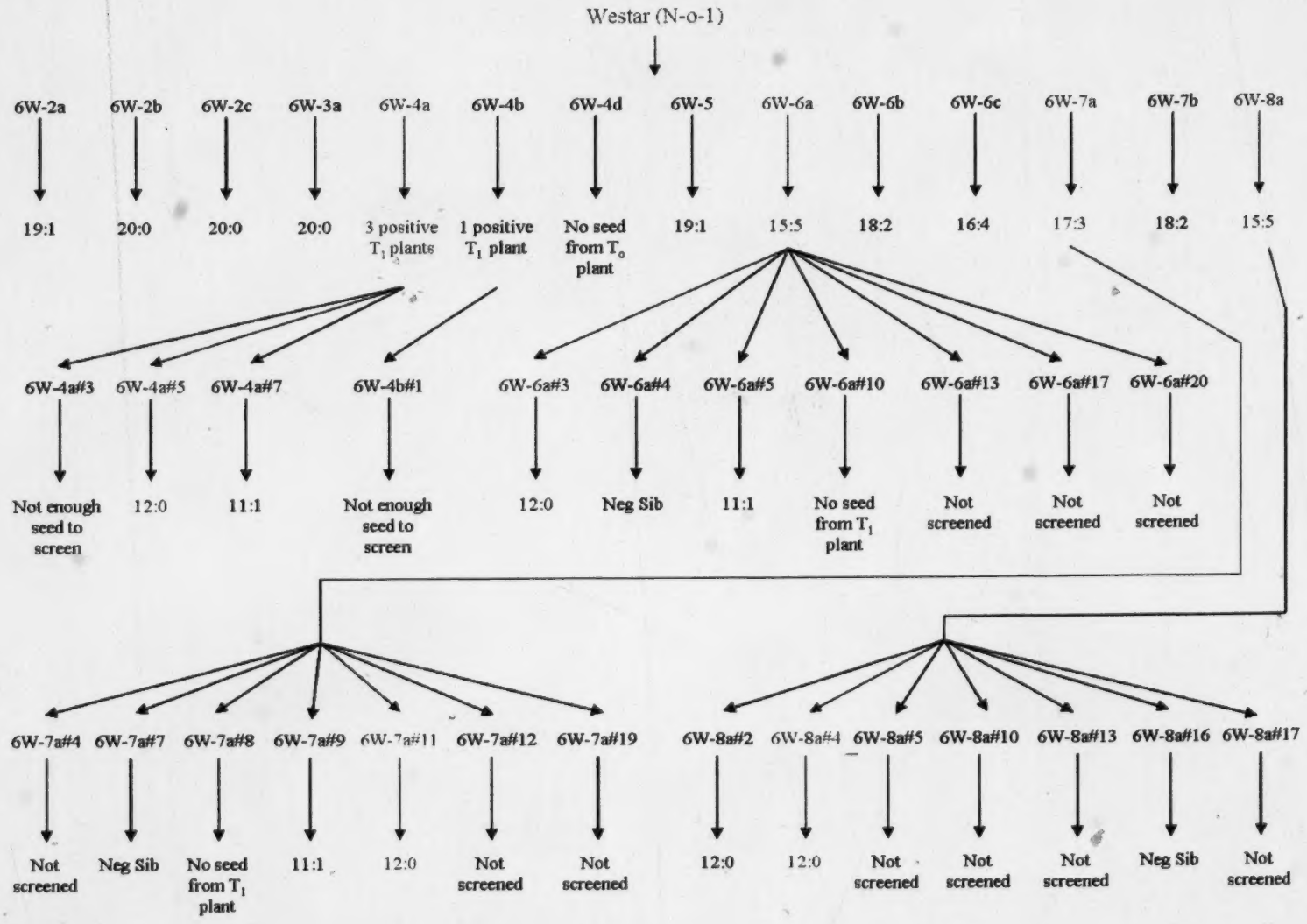
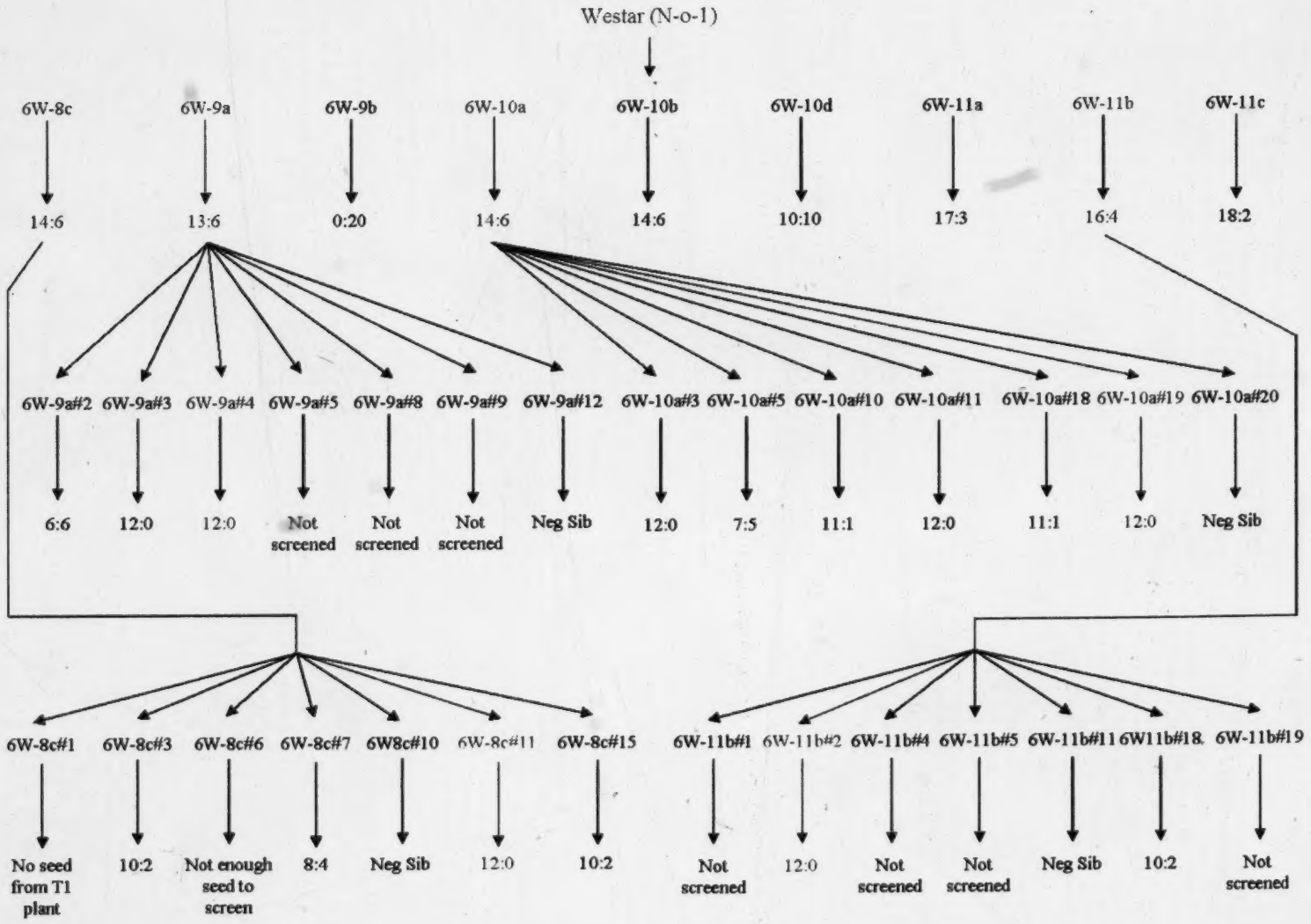
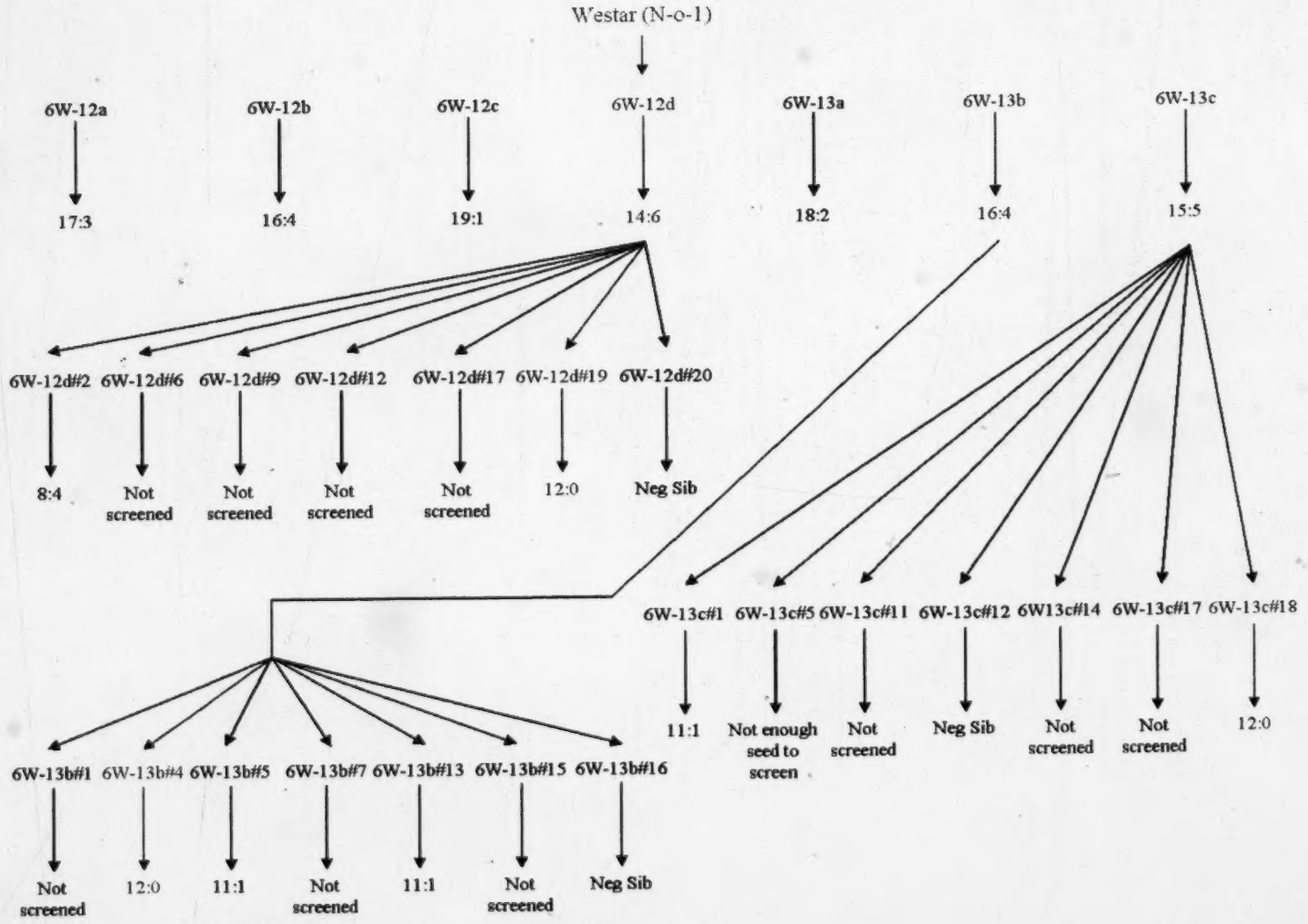
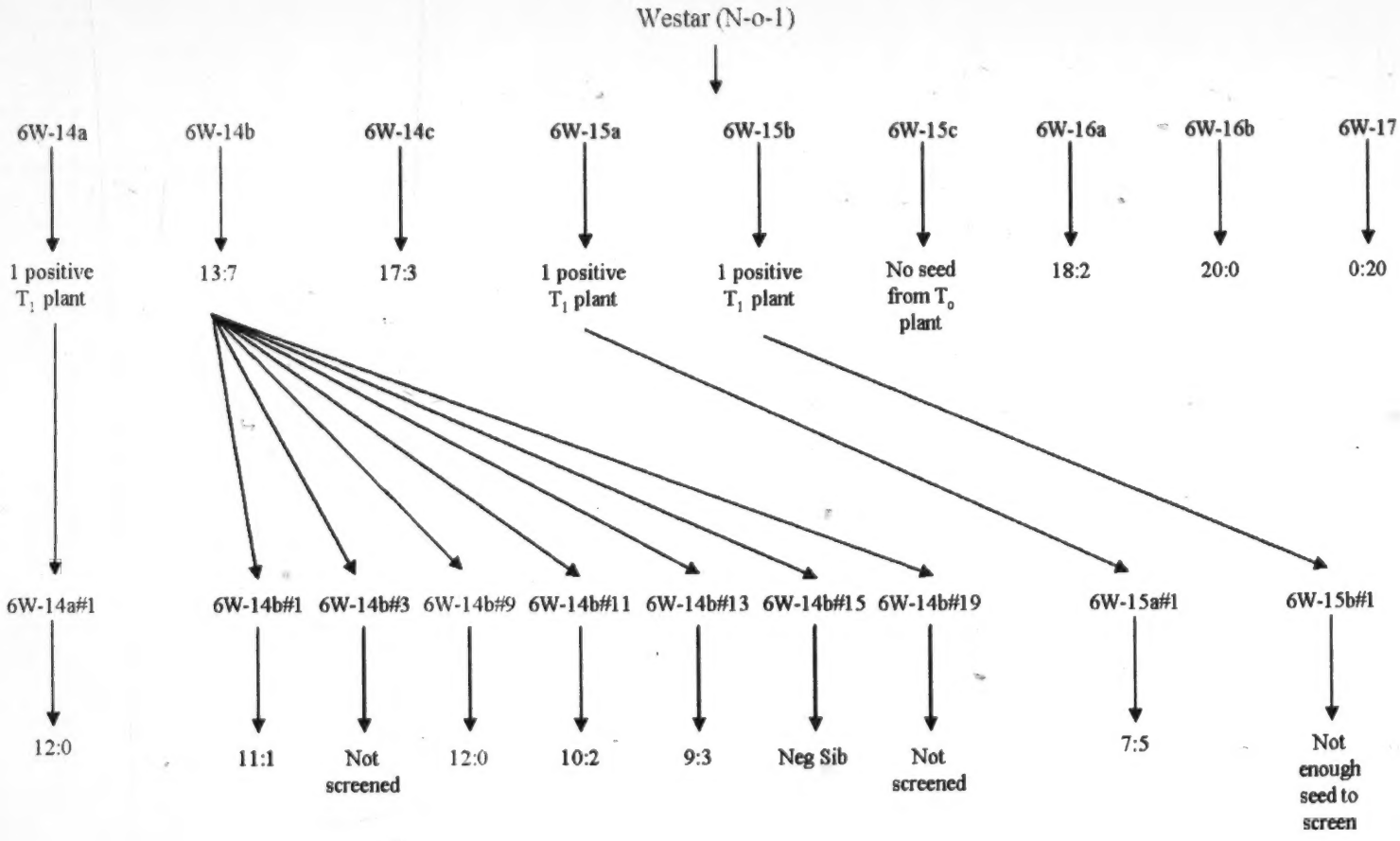
Appendix A: Pedigree of all T_0 transformants that resulted from the *Agrobacterium* mediated transformation of binary vector p26gAspATNS47 into *Brassica napus* cv Westar. Ratios below an arrow are the number of plants that were PCR positive for the transgene to those that were PCR negative for the transgene. Lines with a 3:1 ratio in the T_1 generation were selfed, advancing them to the T_2 generation. Lines containing all positive T_2 plants were again selfed in order to create a T_3 generation of plants homozygous for the transgene. Red arrows and text indicate a transgenic line carried all the way to the T_3 generation and blue arrows and text indicates the transgenic lines corresponding to the non-transgenic negative sibling. Details of the PCR screening procedure used can be found in section 2 of this thesis.


Westar (N-o-1)
6W-2a
6W-2b
6W-2c
6W-3a
6W-4a
6W-4b
6W-4d
6W-5
6W-6a
6W-6b
6W-6c
6W-7a
6W-7b
6W-8a
19:1
20:0
20:0
20:0
3 positive T1 plants
1 positive T1 plant
No seed from T0 plant
19:1
15:5
18:2
16:4
17:3
18:2
15:5
6W-4a#3
6W-4a#5
6W-4a#7
6W-4b#1
6W-6a#3
6W-6a#4
6W-6a#5
6W-6a#10
6W-6a#13
6W-6a#17
6W-6a#20
Not enough seed to screen
12:0
11:1
Not enough seed to screen
12:0
Neg Sib
11:1
No seed from T1 plant
Not screened
Not screened
Not screened
6W-7a#4
6W-7a#7
6W-7a#8
6W-7a#9
6W-7a#11
6W-7a#12
6W-7a#19
6W-8a#2
6W-8a#4
6W-8a#5
6W-8a#10
6W-8a#13
6W-8a#16
6W-8a#17
Not screened
Neg Sib
No seed from T1 plant
11:1
12:0
Not screened
Not screened
12:0
12:0
Not screened
Not screened
Not screened
Neg Sib
Not screened


Westar (N-o-1)
6W-8c
6W-9a
6W-9b
6W-10a
6W-10b
6W-10d
6W-11a
6W-11b
6W-11c
14:6
13:6
0:20
14:6
14:6
10:10
17:3
16:4
18:2
6W-9a#2
6W-9a#3
6W-9a#4
6W-9a#5
6W-9a#8
6W-9a#9
6W-9a#12
6W-10a#3
6W-10a#5
6W-10a#10
6W-10a#11
6W-10a#18
6W-10a#19
6W-10a#20
6:6
12:0
12:0
Not screened
Not screened
Not screened
Neg Sib
12:0
7:5
11:1
12:0
11:1
12:0
Neg Sib
6W-8c#1
6W-8c#3
6W-8c#6
6W-8c#7
6W8c#10
6W-8c#11
6W-8c#15
No seed from T1 plant
10:2
Not enough seed to screen
8:4
Neg Sib
12:0
10:2
6W-11b#1
6W-11b#2
6W-11b#4
6W-11b#5
6W-11b#11
6W11b#18
6W-11b#19
Not screened
12:0
Not screened
Not screened
Neg Sib
10:2
Not screened


Westar (N-o-1)
6W-12a
17:3
6W-12b
16:4
6W-12c
19:1
6W-12d
14:6
6W-13a
18:2
6W-13b
16:4
6W-13c
15:5
6W-12d#2
8:4
6W-12d#6
Not screened
6W-12d#9
Not screened
6W-12d#12
Not screened
6W-12d#17
Not screened
6W-12d#19
12:0
6W-12d#20
Neg Sib
6W-13c#1
11:1
6W-13c#5
Not enough seed to screen
6W-13c#11
Not screened
6W-13c#12
Neg Sib
6W13c#14
Not screened
6W-13c#17
Not screened
6W-13c#18
12:0
6W-13b#1
Not screened
6W-13b#4
12:0
6W-13b#5
11:1
6W-13b#7
Not screened
6W-13b#13
11:1
6W-13b#15
Not screened
6W-13b#16
Neg Sib


Westar (N-o-1)
6W-14a
6W-14b
6W-14c
6W-15a
6W-15b
6W-15c
6W-16a
6W-16b
6W-17
1 positive T1 plant
13:7
17:3
1 positive T1 plant
1 positive T1 plant
No seed from To plant
18:2
20:0
0:20
6W-14a#1
6W-14b#1
6W-14b#3
6W-14b#9
6W-14b#11
6W-14b#13
6W-14b#15
6W-14b#19
6W-15a#1
6W-15b#1
12:0
11:1
Not screened
12:0
10:2
9:3
Neg Sib
Not screened
7:5
Not enough seed to screen